GROWTH, SPORULATION AND SPORE PROPERTIES OF *BACILLUS STEAROTHERMOPHILUS* PRODUCED IN CHEMICALLY DEFINED MEDIA

A THESIS

submitted by

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SUMMARY

The nutritional requirements for the vegetative growth of B. stearothermophilus strains NCIB 8919, NCTC 10,003 (wild) were found to be DL-methionine, biotin, nicotinic acid, thiamin, glucose and mineral salts. Strains NCIB 8920 required in addition L-tryptophan. B. stearothermophilus NCTC 10,003 (mutant) grew in a medium containing only glucose and mineral salts.

Separate chemically defined media for the growth of Bacillus stearothermophilus strains NCIB 8919, 8920, NCTC 10,003 (wild) and NCTC 10,003 (mutant) were developed.

Optimally aerated culture of B. stearothermophilus NCTC 10,003 (mutant) required 1.0×10^{-4} M. Mn^{2+} and 2.4×10^{-3} M. glutamic acid for optimal sporulation. Specific nutrient depletion of growth affected percentage sporulation.

Spore suspensions of B. stearothermophilus NCTC 10,003 (mutant) were prepared from media in which sulphate (SO_4-), nitrogen (N-), phosphate (PO_4-), carbon (C-), magnesium-carbon simultaneously (Mg-C-) depleted growth. The heat resistance, dormancy and chemistry of these spores varied considerably.

B. stearothermophilus NCTC 10,003(mutant) spores prepared from carbon depleted cultures containing high and low concentrations of calcium, iron or manganese showed variations in heat resistance, dormancy and chemical composition.

Progressive increase in the concentration of medium calcium from 1.0×10^{-5} M. to 1.4×10^{-4} M. progressively increased the heat resistance of B. stearothermophilus NCTC 10,003 (mutant)

spores prepared from nitrogen depleted cultures (N-). The thermodynamic functions for germination rate, magnesium and manganese release of N- and SO_4- spores were within the range expected of enzymic reactions. The thermodynamic functions for the breaking of dormancy in SO_4- spores and that for the release of D.P.A. were identical.

Sublethal heating of SO_4- spores ($96.5^{o}C$ and below) induced dormancy in these spores, whereas heating above $96.5^{o}C$ gave rise to heat activation.

Pooled results of the chemical analyses of all spore types studied showed that the concentration of D.P.A. and calcium were positively related to heat resistance whereas magnesium concentration and Mg/Ca molar ratio were inversely proportional to heat resistance.

CONTENTS

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ABBREVIATIONS

cm.	=	Centimetre
mm.	=	Millimetre
μ	=	Micrometre (micron)
nm.	=	Nanometre
g.	=	Gram
μg.	=	Microgram
M.	=	Molar
mol.	=	Mole
hr.	=	Hour
mn.	=	Minute
sec.	=	Second
D	=	Decimal reduction time
S	=	Svedberg unit
K_m	=	Michaelis constant
^oC or deg.	=	Degree Centigrade
^oK	=	Degree Kelvin
Ea	=	Activation energy
ΔS	=	Entropy of activation
ΔG	=	Free energy of activation
$\sim$	=	Approximately
r	=	Correlation coefficient
P	=	Probability
D.F.	=	Degree of freedom
w.r.t.	=	With respect to
r.p.m.	=	Revolutions per minute
E_{420}	=	Optical density at 420 nm.

k cal.	=	Kilocalories
Ala	=	Alanine
Glu	=	Glutamic acid
D.A.P.	=	Diaminopimelic acid
D.P.A.	=	Dipicolinic acid
P.H.B.	=	Poly-β-hydroxy-butyric acid
R.N.A.	=	Ribonucleic acid
D.N.A.	=	Deoxyribonucleic acid
T.C.A.	=	Tricarboxylic acid
E.D.T.A.	=	Ethylenediamine-tetraacetate
HEPES	=	N-2-Hydroxy-ethylpiperazine-ethane-sulphonic acid

ORIGIN AND SCOPE OF THE STUDY

In recent years, considerable attention has been focused on the possibility of using B. stearothermophilus spores as biological indicators to verify the effectiveness of sterilization processes (Friesen and Anderson, 1974; Anderson and Friesen, 1974; Heintz et al., 1976). The United States Pharmacopeia (U.S.P.) and Scandinavian Pharmacopeia (Heintz et al., 1976) have recommended the use of such tests. Similar recommendation was made in the second Pharmacopeia of the German Democratic Republic (Hora and Madimerth, 1973). However, in the British Pharmacopeia, the use of biological indicators was treated with considerable reservation. This stems from two reasons. First, considerable variation in the performance of commercially available indicators containing B. stearothermophilus spores have been reported (Mayernick, 1972; Macek, 1972). Second, there is the risk of mix-up and contamination that may result from the introduction of spore strips into an otherwise microbiologically clean area.

The need for the production of standardized spores to improve the performance of biological indicators has been suggested by several workers (Costin and Grigo, 1974; Friesen and Anderson, 1974; Henning, 1973; Macek, 1972). The importance of a standardized procedure for spore production, storage and use was pointed out in the U.S.P. XIX. Hodges and Brown (1975) prepared spores of B. megaterium from simple salts media in which glucose alone was depleted (G-) and in which glucose and magnesium were simultaneously depleted (G-Mg-). They demonstrated that spores from the two sporulation media were different

with respect to size, chemistry, heat resistance, rate and extent of germination and rate of release of dipicolinic acid (at 80°C, pH 4). The importance of specific nutrient depletion of vegetative growth upon subsequent spore properties was implicated. In view of this and the wide interest in utilizing B. stearothermophilus spores as biological controls in sterilization processes, it was decided to extend the study of Hodges and Brown (1975) to B. stearothermophilus spores. The properties of spores produced under other nutrient depleting conditions such as nitrogen, sulphate and phosphate were also studied. The effect of using different concentrations of medium components, at non-depleting levels, on the properties of a specific spore type were also examined. The aim was to prepare spores maximally defined with respect to medium composition and the nature of the nutrient depletion.

I N T R O D U C T I O N

1. NUTRITIONAL REQUIREMENTS OF BACILLUS SPECIES

A. SCOPE AND LIMITS OF DISCUSSION

Like all heterotrophs, the ability of Bacillus species to assimilate carbon dioxide gas is limited. Growth of such organism is determined by the substances included in the medium (Guirard and Snell, 1962). These substances include a variety of inorganic ions, appropriate carbon, nitrogen and sulphur compounds from which to synthesize cellular material and an available source of energy to support the synthetic activities. For most bacilli, growth is also determined by the presence of organic substances which the organisms cannot themselves synthesize. These substances include vitamins and amino acids which collectively are known as growth factors.

In this present work, detailed discussion of nutritional requirements will be restricted mainly to the growth factor requirements. The reasons for this are two-fold. Firstly, a search of the literature revealed that the inorganic and other organic requirements of Bacillus species were obtained during studies on factors affecting the sporulation process rather than studies on their requirements for vegetative growth per se and this will be appropriately discussed in Section 2.C. Secondly, this work deals principally with the cultivation of Bacillus species in chemically defined media and it is the growth factors of the medium that determine the ability of most Bacillus species to grow in a complex non-defined medium but not in a simple chemically defined medium.

B. GROWTH FACTOR REQUIREMENTS OF BACILLUS SPECIES

Early studies in this field concerned the growth of insect pathogens. Katznelson and Lochead (1947) successfully cultivated Bacillus alvei in a glucose-salts medium containing thiamin and 14 amino acids. Silverster (1964) grew Bacillus popilliae in a semi-synthetic medium containing a 'vitamin-free', salt-free, acid hydrolysate of casein supplemented with dextrose, DL-tryptone and thiamin. However, when the casein hydrolysate was replaced with a complete array of amino acids, no growth occurred unless barbituric acid was added.

The vitamin requirements of thermophilic Bacillus species were studied by Cleverdon et al.(1949 a). Employing a vitamin-free casein hydrolyzate medium they found niacin, thiamin and biotin were required for growth of Bacillus coagulans at 37°C and 55°C. Growth and sporulation were more abundant at the lower temperature. Extending the study to twelve obligate thermophilic strains of Bacillus stearothermophilus (Cleverdon et al. 1949 b), they found biotin, niacin and thiamin to be essential for the growth of cultures at 55°C and 65°C. One group of five cultures required only biotin and niacin, another group of two cultures required only biotin, and a third group of five cultures required all three vitamins. With the exception of one strain no temperature correlated difference in vitamin requirements was observed. This one strain grew well at 55°C in the absence of biotin although at 65°C it required biotin.

Campbell and Williams (1953) showed that the temperature of incubation affects the nutritional requirements of thermophiles. Table 1 presents some representative data, they classified the organisms into three groups. One group showed no difference in growth requirements regardless of the incubation temperature (B. coagulans 2F). The second group showed additional requirements as the temperature of incubation was increased (B. coagulans 1039 (F); B. stearothermophilus 4259 (F) and B. stearothermophilus 1373 b (0)). The third group showed additional requirements as the incubation was lowered (B. stearothermophilus 3690 F).

TABLE 1

THE EFFECT OF INCUBATION TEMPERATURE ON THE NUTRITIONAL REQUIREMENTS OF THERMOPHILIC BACTERIA

(ADOPTED FROM CAMPBELL AND WILLIAMS, 1953)

ORGANISMS	NUTRITIONAL REQUIREMENTS AT :		
	36°C	45°C	55°C
B. coagulans 2 (F)	Histidine, thiamin, biotin, folic acid.	Histidine, thiamin, biotin, folic acid.	Histidine, thiamin, biotin, folic acid.
B. coagulans 1039 (F)	Thiamin, biotin, folic acid.	Thiamin, biotin, folic acid.	Histidine, methionine, thiamin, nicotinic acid, biotin, folic acid.
B. stearothermophilus 3690 (F)	Methionine, leucine, thiamin, nicotinic acid biotin, folic acid.	Methionine, thiamin, biotin, folic acid.	Methionine, thiamin, biotin, folic acid.
B. stearothermophilus 4259 (F)	Biotin, folic acid.	Methionine, histidine, nicotinic acid, biotin, folic acid.	Methionine, histidine, nicotinic acid, biotin, folic acid.
B. stearothermophilus 1373 b (O)	No growth.	Glutamic acid, histidine, methionine, leucine, biotin.	Glutamic acid, histidine, methionine, leucine, biotin, pyridoxal.

(F) - Facultative thermophile (O) - Obligate thermophile

In all instances, biotin is needed. Sundaram (1973) isolated a prototrophic thermophilic B. stearothermophilus from manure. The bacteria were in a state of biotin insufficiency when cultured in medium consisting of inorganic salts and a carbon source. The entire pyruvate carboxylase was produced as biotin-free, enzymatically inactive apo-enzyme. Supplementation of medium with biotin resulted in a rapid conversion of the apo-enzyme into the active holoenzyme even in the absence of growth and protein synthesis.

The nutritional requirements of vegetative cells and spores were compared by Amaha and Sakaguchi (1952) in Bacillus natto, Bacillus subtilis, Bacillus megaterium I & II, Bacillus mycoides and Bacillus cereus. A total of 18 amino acids were tested. The vegetative cells of all strains and spores of B. subtilis and B. mycoides did not require any of the amino acids. However, B. natto spores required isoleucine, B. cereus spores required valine, B. megaterium I spores required alanine and threonine and B. megaterium II spores required phenylalanine.

Similar studies were undertaken by O'Brien and Campbell (1957) on the nutritional requirements for spore germination, outgrowth and vegetative cell growth of B. stearothermophilus, B. coagulans and B. cereus terminalis. The minimum requirements for germination and outgrowth of spores of B. stearothermophilus at 55°C were isoleucine, leucine, valine, methionine, histidine, arginine, thiamin, nicotinic acid and biotin. The vegetative cell growth requirements were identical, except that leucine and

nicotinic acid were not required. B. coagulans required methionine, thiamin, biotin and folic acid for vegetative growth at 35 and 55°C. For spores to germinate and give outgrowth at 55°C, additional metabolites of glutamic acid, histidine, isoleucine, leucine and valine were required. When germination and outgrowth were carried out at 35°C, leucine was not required. The spores of B. cereus required isoleucine, leucine, valine and methionine at 35°C but the vegetative cells did not require leucine.

These studies, however, do not distinguish between the nutritional requirement for germination and those required for outgrowth. It is likely that they are different. Studies have shown that the requirement for germination are more exacting than those for growth (Hill, 1949) and newly germinated bacilli are also more nutritionally exacting than bacilli formed from vegetative cell divisions (Fulvertaft and Haynes, 1951).

Recently, Atkinson et al. (1975) had demonstrated that the amino acid requirements of B. stearothermophilus depend on the nature of carbon source used for growth. With sucrose as the carbon source, the only amino acids used in appreciable quantity were serine and arginine. When sucrose was replaced by glycerol; glutamate, aspartate and threonine were also used.

B. megaterium is well noted for its simple nutritional requirements. Strains examined previously were found to grow in simple chemically defined medium of glucose and mineral

salts (Knight and Proom, 1950). This ability of B. megaterium had been used as an important means of identification of the species and as a distinction from B. cereus which resembles B. megaterium in many respects but requires amino acids for growth (Wolf and Barker, 1968; Gibson and Gordon, 1975). This view was challenged by White (1972) who showed that out of nine strains of B. megaterium from a culture collection, only five grew in a minimal medium that contained only glucose-mineral salts. One strain required biotin while three other strains required two or more amino acids for growth. A freshly isolated strain of B. megaterium required three amino acids for growth. On the other hand, of the three strains of B. cereus from the culture collection, each strain required two or more amino acids for growth initially. Two of the strains gave rise to substrains independent of amino acids on serial transfers.

C. NUTRIENT DEPLETED GROWTH AND ITS EFFECT ON BACTERIA

(a) Organic Nutrient Depletion

Organic compounds form the bulk of the dry weight of bacteria so that if growth is to take place, utilizable carbon, nitrogen, sulphur and mineral salts must be made available in the medium in relatively high concentration. Carbon is often supplied in the form of sugars (glucose, sucrose, maltose etc.) or some amino acids. Nitrogen is supplied in the form of amino acids or ammonium salts. Sulphur may be included in the form of sulphur containing amino acids (methionine, cystine or cysteine) or inorganic sulphate. The response of bacteria to forced organic nutrient deficiency is interesting. Organic nutrient deficiency often led to an altered metabolism and morphology. These altered characteristics can be observed with a nutrient limited culture in a chemostat or with a nutrient depleted culture as in batch cultivation.

The effect of carbon and nitrogen deprivations on the metabolism of cells have been studied in Escherichia coli (Britten and McClure, 1962; Morris and DeMoss, 1966; Jacobsen and Gillespie, 1968; Irr, 1972) in Aerobacter aerogenes (Kennell and Magasanik, 1964) in Pseudomonas aeruginosa (Kay and Groulund, 1969) and in thermophilic Bacillus (Epstein and Grossowicz, 1969). The list is not exhaustive. Britten and McClure (1962) found E. coli starved for glucose, maintained a highly concentrated amino acid pools for several hours, even though the rate of amino acid uptake by the cells, as measured by ^{14}C-proline incorporation was greatly reduced (20-fold). In the guanine and arginine

requiring double mutant of A. aerogenes, Kennell and Magasanik (1964) reported that the synthesis of protein was only 3% per hour of the total protein in the cells, during guanine starvation. The rate of protein synthesis before starvation began was about 40%. Conceivably, the maintenance of concentrated amino acid pool as observed by Britten and McClure (1962) may be the result of reduced rate of amino acid utilization as also evidenced by the massive degradation of ribonucleic acid (Jacobsen and Gillespie, 1968) and polysomes (Morris and DeMoss, 1966) under these conditions or an increase in protein breakdown (Epstein and Grossowicz, 1969) or both.

Irr (1972) found nitrogen starved cells of E. coli had a 50-fold reduction in the content of 16S and 23S ribonucleic acid accompanied by a severe restriction on nucleotide biosynthesis.

Extending the work of Britten and McClure (1962), Kay and Groulund (1969) found during carbon and nitrogen deprivations of P. aeruginosa, the transport rates of glutamate, alanine and glycine in fact increased whereas that of leucine and valine were decreased. The amino acid pool underwent rapid degradation due to the action of constitutive amino acid catabolic enzymes. This enzymic activity was interpreted as providing P. aeruginosa with a selective advantage for survival during condition of carbon or nitrogen deprivation.

Very often, carbon and nitrogen deprivation also leads to altered morphology and ultrastructure of the organism (Caslavska et al. 1972; Baker and Park, 1975). Caslavska et al. (1972) studied

the effect of prolonged cultivation of B. subtilis in a chemostat under nitrogen limited condition. They found bacillary rods were transformed into irregular spherical forms. These spheres contained 30% less protein, 65% less lipids and 120% more polysaccharide. The cell wall of the spherical cells lacked the three layered structure characteristics of the bacillary rods and the periplasmic space was not well defined. The sphere wall was directly adjacent to the protoplast, diffused, thicker and irregular. They also lost the ability to form regular septa and to divide.

Baker and Park (1975) showed that Vibrio species existed as spheres after growth had ceased due to the depletion of the carbon/energy source during batch cultivation. The spheres were not viable and arose from degradation, but not complete removal of the murein present in the walls. The spheres also contained less nucleic acid and low molecular weight cytoplasmic constituents than did the characteristic rod shape organisms.

In batch cultivation, nutrient depletion lead to cessation of growth and the maximum cell density is predictable (Brown and Hodges, 1974). For sporulating organisms under appropriate physical and chemical environment (see later), cessation of growth would characteristically result in the formation of spores. The properties of B. megaterium spores formed under condition of nutrient depletion were studied by Hodges and Brown (1975). Spores formed under condition of glucose depletion (G-) were considerally smaller than spores formed from media depleted of both

glucose and magnesium (G-Mg-). G- spores contained less dipicolinic acid, calcium and magnesium. They germinated more rapidly and extensively than did G-Mg- spores without prior heat activation in a phosphate buffered medium of glucose and L-alanine. G- spores gave straight line exponential kill during heat inactivation whereas a shoulder was observed in the initial period of log survivors/time plot of G-Mg- spores.

Starvation for methionine was studied by Bouchie (1973) in a temperature-sensitive mutant of E. coli. Under this condition, deoxyribonucleic acid extracted from cells was reported to be a mixture of half-methylated and unmethylated products. Methionine starvation affects chemotaxis in Salmonella typhimurium, the characteristic tumbling was eliminated in the wild type but failed to do so in an unco-ordinated mutant (Aswad and Koshland, 1974).

Biotin deficiency in Arthrobacter globiformis led to the development of an aberrant cell form and impaired cell division (Chan et al., 1973). In B. stearothermophilus, the entire pyruvate carboxylase was synthesized in an enzymatically inactive apoenzyme form (Sundaram, 1973).

(b) Inorganic Nutrient Depletion

Magnesium ion is important in at least two major categories of metabolic activity. First, it is the most common metal cofactor for those enzymes for which metal requirements have been determined (Vallee, 1960). Second, magnesium is concerned with the stability of ribosomal particles in cell extracts (Chao, 1957) as well as in

growing bacteria (Kennell and Magasanik, 1962). Consequently, magnesium deficiency in bacteria is by far the most widely studied of all the ion deficiencies.

During magnesium starvation of E. coli B, most of the ribosomes breakdown to low molecular weight components but the cells remained viable (McCarthy, 1962). In A. aerogenes (Kennell and Kotoulas, 1967), the major nucleic acid and protein components continued to be synthesized at slower rates during magnesium starvation. Because the ribosome content of the cells did not increase during this period, the macromolecular composition of the cells became increasingly abnormal with time.

Brown and Melling (1968) and Gilleland et al. (1974) found ultrastructural and chemical alteration of the cell membrane following the growth of P. aeruginosa in magnesium deficient medium, these cells were also resistant to lysis by ethylene-diaminetetra-acetate (E.D.T.A.). Alteration of plasma membrane following magnesium starvation was also reported in E. coli (Fill and Branton, 1969). In nutritionally complete exponentially growing cells, network arrangement of particles 2 to 6 nm. in diameter could be seen in the cell membrane. During magnesium starvation, a paracrystalline particle pattern appeared on the membrane and large areas devoid of particles were observed. However, there was no decrease in the magnesium content of the cell envelope per se even after 24 hours of deprivation.

Magnesium depleted culture of B. megaterium failed to sporulate

(Brown and Hodges, 1974). Spores were formed when the cultures were simultaneously depleted of magnesium and glucose (Mg-G-) and such spores differed from G- spores in size, heat resistance and germination characteristics (see above).

The effect of phosphate deficiency on the morphology and wall composition of bacteria were studied by several workers. Ellwood and Tempest (1969; 1972) found that following phosphate limitation of B. subtilis and B. licheniformis in chemostats, teichuronic acids which are sometimes component of cell wall of cells from batch cultures completely replaced the teichoic acid. With B. subtilis, if the growth conditions were changes from phosphate limitation to magnesium limitation, teichuronic acids were lost from the wall and teichoic acid reappeared in their place. This and the reverse change occurred at a rate substantially faster than the rate of biomass synthesis. Forsberg et al. (1973) showed that B. licheniformis mutant with no teichuronic acid or glucose in the wall when phosphate limited in a chemostat, ceased to make teichoic acid and the wall contained a greatly increased proportion of murein. Under this condition, the bacterium appeared as irregular sphere which changes back to a rod when phosphate was resupplied.

Phosphate starvation of P. aeruginosa led to extensive ribosomal degradation (Hou et al., 1966). This could conceivably be the result of increase in the activities of ribonuclease, phosphodiesterase and phospho-monoesterase (Horiuchi, 1959)

Phosphate starved cells of E. coli also lost viability more quickly (80% in 2 days, Mallette et al., 1964) than glucose starved cells (35% in 2 days, McGrew and Mallette, 1962).

The effect of deficiency of other ions have been less widely studied. Sulphate or potassium depletion was reported to slow down the synthesis of protein (Roberts et al., 1963; Lubin and Ennis, 1964). Potassium depleted cells of E. coli were found to accumulate 'K^+-depletion particles' which got rapidly converted to 30S and 50S ribosomes on transfer to medium supporting growth (Ennis and Lubin, 1965). Depletion of manganese from the growth medium of Brevibacterium ammoniagenes caused unbalanced growth death (Oka et al., 1968). Deoxyribonucleic acid synthesis was stopped at the level corresponding to one-fourth to one-third that of the medium supplemented with manganese. On the other hand, cellular ribonucleic acid and the protein syntheses were unaffected. The cells showed unbalanced growth death after 10 hours of depletion. They lost the ability to form colonies while the cell mass was increasing. The elongated cells (bulbous, club-shaped etc.) finally lyzed. Calcium was quoted as required for the growth of Bacteriodes and omission from the medium reduced the growth of these bacteria (Calwell and Arcand, 1974).

2. SPORULATION OF BACILLUS SPECIES

A. SCOPE AND LIMITS OF DISCUSSION

The ability of bacteria to sporulate is largely confined to the genus Bacillus and Clostridia which have the genetic potential to undergo this complex change. The conversion of an active vegetative cell into a dormant spore proceeds through a series of morphological, physiological and biochemical changes. Consequently, this complicated process has been studied from the morphological standpoint (Fitz-James and Young, 1969; Walker, 1970) or the genetical standpoint (Hoch and Spizizen, 1969; Young and Wilson, 1972) or the biochemical and physiological standpoints (Buono et al., 1966, Tono and Kornberg, 1967). Thus, it is not appropriate to deal in detail with all aspects of sporulation in this thesis. This section will deal primarily with the induction of sporulation and the environmental factors that influence sporulation and the quality of spores formed.

B. INDUCTION OF SPORULATION

Grelet (1946; 1957) interpreted the induction of sporulation following active growth as the depletion of some factors necessary for vegetative growth. The importance of nutrient depletion on the initiation of sporulation was illustrated by experiments in which nutrient depleted cultures under appropriate conditions sporulated. Replenishment of depleted cultures with fresh medium lead to resumption of vegetative proliferation and suppression of sporulation (Vinter, 1969).

Knaysi (1948) suggested that the cells must be in a "healthy state" before sporulation could ensue. Brown and Hodges (1974) found that magnesium depleted cultures of B. megaterium did not form spores whereas cultures simultaneously depleted of magnesium and glucose did. It is not known whether the culture depleted of magnesium and glucose simultaneously is any "healthier" than the glucose depleted culture.

Hutchison and Hanson (1974) and Hanson (1975) reckoned that a temporary depletion of energy source is necessary for the initiation of sporulation. They found the amount of adenosine triphosphate per cell in sporulating cells was approximately half that observed with cells leading to vegetative cell divisions.

Schaeffer et al. (1965) suggested that sporulation is repressed by some intracellular nitrogen containing metabolites (see below). Since then, many sporulation enzymes (notably tricarboxylic acid cycle enzymes) catabolically repressed during vegetative growth have been reported and several workers (Cox and Hanson, 1968; Fletcher and Hanson, 1969) suggested that the exhaustion of these catabolites as a necessary pre-requisite for these enzymes to be derepressed, giving rise to sporulation. These workers have suggested that growth limitation by an energy source, nitrogen, phosphate or adenosine would result in a release from catabolite repression, whereas growth limitation by sulphur or tryptophan would increase the severity of catabolite repression.

The suggestion that sporulation in bacteria is a response

of cells to the depletion of nutrients (energy source) necessary for growth, however, could not account for some of the reported observations with B. megaterium and B. subtilis, that formed a low proportion of spores in cultures even during exponential growth (Aubert et al.,1961; Kerravala et al., 1964; Schaeffer et al., 1965). From observations of batch cultures growing at maximal growth rates, Schaeffer et al. (1965) calculated a 'probability for a cell to become committed to sporulate' in a wide variety of minimal media. They found that this probability which defines the decision to sporulate rather than grow was relatively independent of the growth rate of the culture, but depends on the carbon and nitrogen sources used for growth. They proposed that sporulation was repressed by some intracellular nitrogen containing metabolites.

Hanson (1966) suggested that sporulation was the response of a genetically apt bacterium to an increase in the generation time. This was seen partly to be the case of B. subtilis cultivated continuously (Daves and Mandelstam, 1970). These workers found that the incidence of spore formation increased with decrease in the growth rate of the culture.

The nutrient depletion concept also could not account for the observation that sporulation can be induced in vegetative cells by limited lesions in the cell wall (see Microcycle Sporulation, Section 2.F.) or the formation of spores following incubation with an endogenous factor (Srinivasan and Halvorson, 1963). Conceivably, the decision to sporulate may well be under the control of some

"sporogenes" and the genome responsible for sporulation could be triggered by damage generated in the organisms, like lesions of cell walls or metabolic damage following energy depletion. An "operon model" type of genetical control described by Jacob and Monod (1961 a; 1961 b) have been suggested by several workers (Srinivasan, 1965; Kornlohr and Leitzmann, 1969).

C. THE EFFECT OF ENVIRONMENTAL CONDITIONS ON SPORULATION

(a) Physical Factors

(i) Temperature

The temperature of cultivation affects both the rate and extent of sporulation and also the quality of spores formed. Sporulation occurred very rapidly at or near optimal growth temperature and the extent of spore yield was reduced by growth at unfavourable temperatures (Migula, 1904). The importance of appropriate incubation temperature on spore formation is illustrated by the finding of Hardwick and Foster (1952), who showed that sporulation of B. mycoides was interrupted by rapid cooling to 4°C. Prompt sporulation took place as soon as the culture was returned to 30°C, even after a month storage.

A general correlation of heat resistance with the temperature of sporulation was shown by Williams and Robertson (1954). They showed that various facultatively and obligately thermophilic strains of B. stearothermophilus responded to increase in temperature of sporulation by producing spores of increased thermoresistance. Similiar results were obtained by Friesen

and Anderson (1974) using a single strain of B. stearothermophilus at the temperature range of 50-65°C.

Elevation of the sporulation temperature of B. subtilis led to increase in dipicolinic acid and calcium contents of spores, with corresponding increase in heat resistance. With the thermophile, the opposite was found. B. coagulans spores had lower content of dipicolinic acid as the sporulation temperature was increased (Lechowich, 1959).

(ii) Oxygen

Sporulation in aerobic bacilli has long been known to require oxygen (Weil, 1899). Roth et al. (1955) found in mutants of B. anthracis and B. globigii, that the process of sporulation required about five fold more oxygen than did vegetative growth. Generally, the oxygen demand is higher in the prespore stage, demand decreases rapidly as sporulation proceeds to completion (Hardwick and Foster, 1952; Halvorson, 1957). In B. megaterium (Slepecky and Law, 1960), the higher oxygen demand was reported to be required for the oxidation of poly-B-hydroxybutyric acid and the ability of cells to sporulate was correlated with this oxidative ability.

Nevertheless, there are reports that showed that excessive oxygenation is detrimental to the spore formation process. Grelet (1952) reported the isolation of a mutant of B. megaterium which was induced to sporulate only when the medium was oxygen limited. In a strain of B. stearothermophilus (Long and Williams,

1960), sporulation was reported to be oxygen sensitive at 55°C but not at 37°C. Remarkedly, the oxygen demand for sporulation of this organism was also influenced by the nature of the inoculum. When the culture was initiated from spores, a high rate of aeration promoted growth and sporulation. If vegetative cells were used as the inoculum instead, high aeration allowed vigorous vegetative growth and sporulation was inhibited.

(iii) pH

During batch cultivation, the pH and oxygen demand of the culture dropped at the exhaustion of glucose. This was followed by a sharp rise in the oxygen demand and coincided with a rise of pH and initiation of the pre-sporulation stage (Halvorson, 1957). In general, the tolerance of growing cells to pH changes is much higher than that of sporulating cells (Leifson, 1931). The pH optimum for sporulation of most bacilli is close to neutral, narrower than that required for vegetative growth (Murrell, 1961). However, B. coagulans var thermoacidurans was reported to sporulate optimally at pH 5.5 (Amaha et al., 1956).

(b) Chemical Factors

(i) Organic components of medium

A variety of organic nutrients have been reported to stimulate sporulation. They include glucose (Foster and Heiligman, 1949); amino acids (Williams and Harper, 1951); vitamins (Lund et al., 1957); inositol(Hayward, 1943) and organic acids (Amaha et al.,

1956). The validity of these claims is difficult to evaluate because different organisms are used in each case. Also, most investigators did not distinguish between factors influencing vegetative cell growth and those affecting sporulation. Using chemically defined medium, Grelet (1957) showed that some of these factors were in fact inducers of sporulation only when depleted during growth. The suppression of sporulation by organic nutrients and the depletion of these nutrients on the induction of sporulation have been discussed above (see Section 2.B.) Nevertheless, there are evidence that showed some amino acids e.g. glutamic acid, isoleucine, threonine and valine must be present in the medium for sporulation to take place (Bernlohr, 1965; Buono et al., 1966 Kennedy et al., 1970; Doering and Bott, 1972).

In B. cereus, Buono et al. (1966) found that a high level of glutamic acid (70 mM.) was required for optimal growth and glucose oxidation followed by sporulation even though relatively little glutamic acid was consumed (14 mM.).

Sporulating cells of B. licheniformis (Bernlohr, 1965) were reported to develop increased ability to oxidize glutamic acid, isoleucine, threonine, valine and several other amino acids concomittant with the cessation of growth.

The exact role of glutamic acid is not clear. Glutamic acid was reported to act as an amino donor in sporulating cells (Siegenthaler and Hermier, 1964), as a precursor to dipicolinic acid biosynthesis (Martin and Foster, 1958) and as a carbon and

nitrogen substrate for the syntheses of amino acids during the transition of a vegetative cell to a spore (Bernlohr, 1965). Nickerson et al. (1972) found that glutamic acid regulated the tricarboxylic acid (T.C.A.) cycle activity in Bacillus thuringiensis. Cells cultured in medium with low glutamic acid concentration lacked a functional T.C.A. cycle as judged by radiorespirometric study on glutamin -2 ^{14}C, -3, 4 ^{14}C and -5 ^{14}C or acetate -1 ^{14}C, -2 ^{14}C when virtually no $^{14}CO_2$ was released from either substrate. On the other hand, cells grown in high glutamic acid released $^{14}CO_2$ thus, was consistent with the fact that glutamic acid oxidation occurred via an intact T.C.A. cycle. Moreover, the spores from low glutamic acid medium were sensitive to heat shock and had a reduced dipicolinic acid content compared with spores formed from high glutamic acid medium. It was suggested that perhaps, a functional T.C.A. cycle is essential to the synthesis of dipicolinic acid by providing aspartate as a precursor.

During sporulation, the cells showed differential amino acid requirements(Doering and Bott, 1972). Using auxotrophic mutants of B. subtilis 168, these workers found sporulation was completely inhibited when isoleucine, tryptophan and threonine were omitted. Also, tryptophan was required earlier in the sporulation process than threonine. Isoleucine omission did not affect the early sporulation functions like the protease formation or septum formation, but prevented the increased levels of protein synthesis and oxygen consumption that normally accompany early sporulation stages.

De Guzman et al. (1972) reported that with B. stearothermophilus cultured in chemically defined medium, the percentage sporulation was higher when the amino acids histidine, tryptophan, methionine or lysine were used individually rather than in combination. It is not clear whether the amino acids quoted were used as major nutrients or they were incorporated into the medium as growth factors.

Some observations that the nature of nutrient depletion affects the rapidity of spore formation is provided by the study of Dawes and Mandelstam (1970) in continuous culture. They found cultures of B. subtilis was immediately committed to sporulation as soon as they were initiated by glucose limitation, whereas with nitrogen limited cultures, a partial relief of nitrogen during the first hour after initiation prevented the development of spores.

Hitchins et al. (1972) found that the size and chemical composition of B. megaterium spores were dependent on the nature of carbon source used. Small, spherical spores were produced after batch cultivation in citrate whereas large, oblong spores were produced by replacement in the presence of acetate. Large and small spores had approximately the same amount of deoxyribonucleic acid, density and heat resistance, but the large spores had more dipicolinic acid, glucosamine, ribonucleic acid, manganese, lipid, iron, calcium, zinc and dry weight.

(ii) The inorganic components of medium

The importance of manganese for sporulation was first

demonstrated by Charney et al. (1951) in B. subtilis. Using chemically defined and complex organic media, they found sporulation was negligible without added manganese. This observation was later confirmed by Curran and Evans (1954). They also observed that iron when used in relatively large amount could replace the manganese requirement. However, it was reported later (Curran, 1957) that when a sample of ferric chloride which in nutrient broth promoted characteristic sporogenic activity in B. subtilis was purified repeatedly with hydrochloric acid and ether, the sporogenic activity was lost even though the medium contained equal amount of iron as before. The spore inducing property of the medium containing purified iron could be restored by the addition of manganese.

Weinberg (1964) studied the manganese requirement for sporulation of a laboratory strain of B. megaterium and found that considerably higher concentrations of the manganese were required for sporulation than for normal vegetative growth over the manganese range of 0.01×10^{-6} M. to 10×10^{-6} M.

In B. coagulan var thermoacidurans, manganese had been reported to broaden the temperature and pH range over which sporulation could occur (Amaha et al., 1956)

The manganese concentration in the medium also affects the quality of spores formed (Tallentire and Chiori, 1963; Levinson and Hyatt, 1964; Gruft et al., 1965; Aoki and Slepecky, 1973). Tallentire and Chiori (1963) prepared spores of B. megaterium in

medium containing only Mg^{2+} and Fe^{2+} as sole divalent metallic cations. Spores were also prepared in this medium to which Mn^{2+} or Ca^{2+} or both had been added or in which Mn^{2+} and/or Ca^{2+} had replaced Fe^{2+}. They found spores prepared in medium containing only Mg^{2+} and Fe^{2+} were the least resistant to heat. The addition of Ca^{2+} to the medium had no effect on the heat resistance of spores; the addition of Mn^{2+} or of (Ca^{2+} plus Mn^{2+}) to the sporulation medium produced spores of intermediate heat resistance. The most heat resistant spores were prepared when Fe^{2+} was replaced by (Ca^{2+} plus Mn^{2+}).

Aoki and Slepecky (1973) studied the effect of manganese levels in sporulation medium on the heat resistance of Bacillus fastidiosus spores. Decimal reduction time at 85°C (D_{85}) of 6.5 minutes and 17.0 minutes were reported for spores grown without added manganese and in the presence of 10^{-5} M. manganese respectively. Moreover, spores prepared from medium with manganese required heat activation for germination. The effectiveness of heat activation was directly dependent on the concentration of manganese in the growth medium.

Gruft et al. (1973) found B. megaterium spores prepared in manganese deficient medium germinated slowly. In the same bacteria, Levinson and Hyatt (1964) showed that spores prepared from medium relatively high in $MnCl_2$ (0.1 mM.) germinated optimally in L-alanine but not in glucose.

Potassium has long been recognised as important for sporulation (Foster and Heiligman, 1949). Eisenstadt and Silver (1971) found

sporulating cells of B. subtilis maintained a high level of internal potassium. Non-sporulating stationary phase cells had low internal potassium due to reduced rate of potassium accumulation coupled with a rapid efflux of potassium. The maintenance of high internal levels of potassium was essential for protein synthesis and was characteristic of both vegetatively growing and sporulating organisms. They found that the presence of manganese in the medium was specifically needed for the accumulation of internal potassium to the sporulation level.

The effect of ion depletions on sporulation was studied by Grelet (1951; 1952). He found sporulation of B. megaterium remained at high levels with progressive reduction in NO_3^-, SO_4^{2-}, Cl^-, Na^+, Fe^{2+} and Zn^{2+} levels. However, when the medium was depleted of potassium, magnesium or manganese, the sporulation capacity of most bacilli was prevented. A deficiency of calcium and phosphate in the medium was reported to reduce the number of spores formed and omission of calcium from the medium did not affect vegetative growth but greatly hindered sporulation and yielded spores slightly refractile and weakly thermoresistant. When calcium was omitted and replaced with an equal concentration of manganese, sporulation was normal but the number of thermoresistant spores was reduced by 90%. Also, when both calcium and zinc were omitted, sporulation was prevented.

Calcium is also required in distilled water for the formation of normal, heat resistant, dipicolinic acid containing spores by the endotrophic process (see below). The importance of calcium

in the medium in determining heat resistance was confirmed in B. subtilis (Donnelian et al., 1964) and in B. stearothermophilus (Friesen and Anderson, 1974). Using ^{45}Ca, Halvorson and Howitt (1961) and Cooney and Lundgren (1962) showed that the uptake of calcium in B. cereus was closely associated with sporulation and dipicolinic acid synthesis. Black et al. (1960) showed that the amount of dipicolinic acid synthesized during endotrophic sporulation of B. cereus was a function of the calcium content in the medium. Different levels of calcium in the medium may also influence the germinative properties of aerobic spores (Levinson and Hyatt, 1964; Donnelian et al., 1964). Levinson and Hyatt (1964) showed that an increase in the level of calcium during sporulation of Bacillus megaterium yielded spores with increased germination ability in glucose but not in L-alanine.

The enhancing effect of calcium on spore heat resistance and germination was reported to be suppressed by the presence of excessive phosphate (El Bisi and Ordal, 1956) or sodium (Fleming and Ordal, 1964). El Bisi and Ordal (1956) suggested that the repression of heat resistance of B. coagulans var thermoacidurans spores produced in high concentration of phosphate was due to the formation of calcium phosphate complex, rendering the calcium less readily available for spore formation. Moreover, the germination rate of B. megaterium spores in glucose was reduced when spores were produced in medium containing high concentration of phosphate (Levinson and Hyatt, 1964).

The importance of trace metals in sporulation was studied in

B. megaterium using purified sucrose-mineral salt medium (Kolodziej and Slepecky, 1964). They found copper, iron and zinc to be indispensible for sporulation. However, unlike manganese, their requirement could be satisfied if adequate amount of molybdenum ions was present. Iron was reported to be utilized by B. cereus during early growth and returned to the medium at the end of sporulation (Cooney and Lundgren, 1962). This observation is in contrast to that of Powell and Strange (1956), they found an increase in the iron content of cells of B. cereus and B. subtilis after sporulation. Other trace metals reported to enhance sporulation include cobalt and nickel (Amaha et al., 1956) and lithium (Ward, 1947).

D. BATCH AND CONTINUOUS CULTIVATION OF SPORES

For sometime, depletion of essential nutrients from the medium had been regarded as a pre-requisite before sporulation could occur. Consequently, most of the data on sporulation have been obtained by the use of conventional batch cultivation technique, in which the sporulation medium contained a limited concentration of essential nutrient, usually a carbon/energy source. Under this condition, the composition of the medium is being gradually changed as growth progresses. This change in the chemical makeup of the medium will undoubtedly affect the properties of spores, formed at the exhaustion of the limiting nutrient. The response of sporulation process and spore properties to changes in the chemical environments has been discussed in Section 2. C. (b).

Later, it was found that some species of bacilli formed spores even during logarithmic growth (Schaeffer *et al.*, 1965; Bashnage-De Pamphilus and Hanson, 1969) and the ability of a given specie to sporulate under this condition has been attributed to the hypothetical difference in the rate of synthesis of the "endogenous sporulation factor" (Srinivasan and Hanson, 1963; 1965). The desirability of synchronization of culture for physiological studies and for the production of spores of consistent properties prompted many workers to explore the use of continuous cultivation.

One of the earliest user of continuous culture method for studying sporulation was Aubert *et al.* (1961), they showed that sporulation of *B. megaterium* was reduced by decreasing the growth

rate from 0.7 to 0.5 division per hour. Later, Kerravala et al. (1964) use the technique to compare the sporulation of B. subtilis and B. cereus T. They found sporulation with B. subtilis but not in B. cereus T which only sporulates at the depletion of nutrient.

Dawes and Mandelstam (1970) showed that B. subtilis sporulated at high frequency when growth limited by glucose or the nitrogen source in minimal medium; whereas rates of sporulation were low for magnesium, phosphate, citrate or tryptophan limitation. Even though sporulation occurred at all growth rates, the incidence of spores increased with decrease in the growth rate of the culture. The theoretical treatment of sporulation during continuous cultivation was also explored by Dawes and Thornley (1970). Sporulation was considered as a probability event and an equation which relates the initiation of sporulation to dilution rate of the culture was described.

Though cultivation of non-sporing species in a chemostat is relatively simple, the cultivation of spore forming bacteria is difficult. Difficulties like the selection of asporogenous mutants (Aubert et al., 1961), failure of some spore-formers to attain a steady state (Kerravala et al., 1964), the germination of all but 10% of the spores formed (Powell, 1970) have been quoted. For some reason, some sporeformers also failed to sporulate in a single stage continuous cultivation system. Malek et al. (1953) used a multi-stage continuous culture method for growth and sporulation of Bacillus pumilus in casamino acids medium. At a suitable dilution rate, a constant fraction of sporangia were

formed. The ratio of vegetative cells to sporangia decreased in the second and third vessel of the cultivation system. The cells were reported to grow in chains and individual cells within the chain showed high variability in their ability to sporulate. Also, the equilibrium between the number of vegetative, granular and sporulating cells was sensitive to the dilution rate. Humphrey et al. (1966) pointed out that B. stearothermophilus spores could not be produced even in a two-stage continuous cultivation system due to the extraordinary lysis of the culture during cultivation. It should be pointed out these workers used complex medium of glucose-yeast extract-mineral salts for cultivation. The nature of growth limitation was not determined. Studies with B. stearothermophilus NCIB 8919 and B. stearothermophilus NCTC 10,003 (mutant) had shown that growth depletion by a carbon and energy source ultimately led to cell lysis whereas growth depletion by other nutrients (nitrogen, vitamins, ions) did not. It seems therefore that the lysis of cells as observed by Humphrey et al. (1966) may be prevented by using appropriate nutrient such as nitrogen, vitamins or ions as limiting nutrient.

The advantages of using continuous culture for spore production (if possible) are enormous. First, sporulating population can be maximally defined with respect to the chemical composition of the medium (see Tempest, 1970). This is a very important consideration from the viewpoint of producing spores of consistent and reproducible heat resistance for use as biological indicators. One of the serious disadvantages with batch cultivation is the continuous alteration of the chemical composition of the medium as growth

progresses. This is not so with continuous cultivation, the chemical composition of the medium remain constant on the attainment of steady state. Since spore properties are determined by the chemical makeup of the medium during sporulation, the importance of maintaining a constant environment during sporulation is self-evident. Second, continuous culture method has proved to offer distinct advantage over batch cultivation in as far as the output of cells is concerned.

E. ENDOTROPHIC SPORULATION

The ability of vegetative cells of sporeformers to sporulate when suspended in distilled water was first demonstrated by Buchner in 1890. This work was later confirmed and extended by Schreiber in 1896, who concluded that spore formation followed the sudden hinderance of growth after adequate nutrition. Hardwick and Foster (1952) called this process "endotrophic sporulation" meaning that it occurred independently of exogenous nutrition and supported exclusively by the pre-existing makeup of the vegetative cell. This suggestion assumed that the synthesis of protein and nucleic acids during sporulation occurred *de novo* from low molecular weight precursors originated by previous degradation of pre-existing macromolecules. They also found that vegetative cells of many *Bacillus* species, when removed from the growth medium before the point of maximum growth and the onset of sporulation and then transferred into distilled water or phosphate buffer sporulated after incubation for 10 hours. The sporulation events began on the eighth hour and took two hours to completion. Normally, about

90% of the cells would sporulate when suspended in water. If glucose was added within the first 5 hour after the cells were transferred into distilled water, subsequent sporulation was completely suppressed. Glucose added to the cells after 6, 7 or 8 hours was progressively less effective and when added after 8 hours was totally ineffective in suppressing sporulation. It was concluded that the addition of glucose up to the sixth hour would cause the reversal of sporulation events, while these events were irreversible when glucose was added after the sixth hour. The cells after the sixth hour were said to be "committed" to sporulation.

Szulmajster (1973) defined commitment as the "point de non retour", the time at which the biochemical and morphological events associated with the developmental system are definitely channelled toward the differentiated form and cannot be reversed.

Commitment to sporulation must be preceded by a period of initiation. However, the two events are distinctively different. A bacterial cell can initiate the sporulation process without being committed to the formation of a mature spore.

With different organisms, the specific point of commitment may vary. B. cereus seems to commit at stage IV (Buono et al., 1966; Fitz-James and Young, 1969). In B. subtilis, the forespore was committed at stage II, but the mother cell was not committed until stage III (Frehel and Ryter, 1969). To some extent, the time and morphological state at which commitment occurred depend on both the medium on which the cells were originally grown and the compounds or media used to replenish the medium (Cooney et al., 1975).

They found with B. megaterium grown in minimal sucrose medium, that commitment occurred earliest in the presence of aspartate or glutamate and next in the presence of fructose, glucose, glycerol or sucrose. Addition of glutamate and a carbohydrate together allowed a later commitment than either compound alone. In a single case where the molecular mechanism is known, B. subtilis became committed to sporulation (as determined by glucose addition) due to a decline in the activity of glucose-phosphoenol-pyruvate transferase and a concomittant inability to transport glucose into the cell (Freese et al., 1970).

Several workers (Powell and Hunter, 1953; Black and Gerhardt, 1962b) had objected to the notion that endotrophic sporulation occurred wholely from the pre-existing makeup of cells. They argued that the lysis of some transferred cells as commonly observed, could convert the water into dilute nutrient medium, which then supported the sporulation of surviving cells. Moreover, in most organisms, notably B. subtilis, endotrophic sporulation occurred only upon the addition of some other substances, a replacement technique (see above, Cooney et al., 1975). Vinter (1959) found that the levels of calcium and cysteine were greater in B. cereus and B. megaterium spores than in the corresponding mother cells and thus could not be derived entirely from intracellular compounds.

Ramaley and Burden (1970) studied the replacement sporulation of B. subtilis 168 in a chemically defined medium. They found that if the substrate was supplied at too high a level or if too few cells were suspended in the sporulation medium, the cells

continued vegetative growth for a short while. On the other hand, if the substrate was supplied at too low a level or the cells were suspended at too high a density for the substrate concentration, the cells lyzed. Optimal sporulation occurred in cells resuspended in medium containing 10 mM ammonium lactate or glutamate, when the inoculum was 2.5×10^8 colony forming units per ml. Also, compounds like ammonium lactate or glutamate that were metabolized slowly by B. subtilis were most effective for use in the replacement medium. They suggested that these compounds probably served as exogenous storage compounds by providing enough energy for sporulation but not enough for growth.

Poly-β-hydroxybutyric acid (P.H.B.) is commonly present in vegetative cells but absent from spores. Tinelli (1955) found that complete oxidation of P.H.B. occurred during endotrophic sporulation of Bacillus species and suggested that this compound supplied the carbon and energy requirements of the process. Later, Slepecky and Law (1962) showed that endotrophic sporulation was only possible with cells having a large reserve of P.H.B.

The physiological changes that occurred during batch growth and sporulation, like the increase in the level of T.C.A. cycle enzymes, changes in the rates of syntheses of deoxyribonucleic acid, ribonucleic acid and protein can also demonstrated in endotrophic sporulation. Other similarities included radioisotope incorporation pattern and the production of normal heat resistant spores (Ramaley and Burden, 1970).

The properties of endotrophically produced spores have been studied by several workers (Black et al., 1960; Pelcher et al., 1963; Foerster and Foster, 1966). Pelcher et al. (1963) prepared spores by a replacement technique using solutions of distilled water, fresh medium, calcium, strontium, barium, nickel, zinc, cobalt and magnesium solutions. They found spores of B. cereus produced in distilled water were heat sensitive and contained low levels of calcium and dipicolinic acid. Heat resistant spores with a normal complement of calcium and dipicolinic acid were formed only in a fresh medium or in solution containing suitable levels (1 mM) of calcium. Of the other metals tested, only strontium stimulated dipicolinic acid synthesis above the level of the spores produced in water.

Foerster and Foster (1966) studied endotrophic calcium, barium and strontium spores of B. megaterium and B. cereus. They found calcium and strontium spores were permanently refractile while a substantial fraction of the barium spores darkened during and after sporulation. There was no anatomical difference among the three types of spores and the different spore types were enriched specifically in the metal to which they were exposed during sporulation. Strontium and barium spores were heat resistant but only the calcium spores attained maximal heat resistance. The three types of spores also exhibited different rate of germination, with the calcium spores germinated fastest and the barium spores, the slowest. Also, it was only the calcium spores which required heat activation for optimal germination. When the spores were heat treated, release of dipicolinic acid occurred much faster with barium and strontium

spores than with calcium spores.

The technique of endotrophic sporulation and replacement offer many advantages in the study of sporulation process. First, the medium for sporulation can be maximally defined and secondly, it is possible to distinguish which requirements for spore formation may be covered by the pre-existing makeup of the cells and which additional components of the medium determine the quality of spores produced. Sporulation initiated by some experimental manipulations and occurred over relatively short period of incubation should result in reasonable synchronization of the spore population.

The use of endotrophic sporulation for the production of spores of consistent and reproducible heat resistance for use as biological indicator in sterilization processes has not been exploited. It may be possible to prepare physiologically defined vegetative cells by continuous cultivation (Tempest, 1970), these cells can then be induced to sporulate endotrophically in defined system.

F. MICROCYCLE SPORULATION

Vinter and Slepecky (1965) first demonstrated that 100-fold dilution of a complex medium used for the outgrowth of germinated spores of *B. cereus* would induce a 90% sporulation even before the first division had occurred. They termed the process "microcycle sporulation" meaning a shortening of the usual developmental cycle. Later, the process was also reported by Holmes and Levinson (1967) in *B. megaterium*. These workers found that microcycle sporulation could also be induced in chemically defined medium which permitted

no cell division. In the case of B. cereus, phosphate limitation was found to be most effective in inducing microcycle sporulation (Hanson and Mackechnie, 1969). Interestingly, microcycle sporulation could also be induced in germinated spores by exposing B. cereus cells to D-cycloserine or vancomycin (Rodenberg et al., 1972). Both antibiotics caused limited lesions in the cell walls and when added simultaneously induced more than 80% of the cells to sporulate. Also, the pattern of macromolecular synthesis in antibiotic induced sporulation was similar to that which occurred during microcycle sporulation induced by a deficient medium. It appears possible that the genome responsible for sporulation could be triggered by damage in the cell walls of the primary cells acting as a signal. This ability of antibiotic to induce microcycle sporulation deserves further studies. Spores of varied heat resistance may be germinated and the primary cells induced to sporulate by antibiotic under carefully controlled sporulation condition. Antibiotic-induced microcycle sporulation of B. cereus occurred after incubation for 40 minutes. The sporulation event took only 20 minutes to completion (Rodenberg et al., 1972). Sporulation initiated by experimental manipulations and occurred rapidly should result in reasonable synchronization of the spore population. The spores prepared this way may be of greater uniformity in heat resistance. Microcycle spores thus prepared may be used as biological indicator in sterilization processes.

The nutritional requirements for microcycle sporulation have been studied by some workers (Holmes and Levinson, 1967; Mackechnie and Hanson, 1968). Holmes and Levinson (1967) showed that no

divalent metal was required by B. megaterium for the process. The initial spores contained sufficient endogenous supply of metals which was incorporated into the second stage spores. They showed that for optimal sporulation, 10 µM glucose and 3 µM NH_4Cl per mg. spores were needed. Increasing or lowering the concentration of either compound separately impaired or delayed completion of the cycle. Glucose per se was not required for microcycle sporulation. Ionically germinated initial spores formed second stage spores in a medium containing 40 mM sodium acetate plus 1 mM of the following: glucose, ribose, citrate, glutamate, isocitrate, succinate, fumarate or oxaloacetate. Acetate alone did not support second stage spore formation. Moreover, the material released into the medium by the initial spores during the early stages of germination was not required for subsequent sporulation. However, adequate phosphate was needed in the medium for spore formation.

Mackechnie and Hanson (1968) showed that for the microcycle sporulation of B. cereus, phosphate concentrations of 1.0×10^{-5} M. to 2.0×10^{-5} M. were optimal to satisfy the demand for a two-fold increase in deoxyribonucleic acid during the process. Increasing the concentration of phosphate to 10^{-4} M. caused significant division.

As in the case of batch growth and sporulation, sulphate is needed in the microcycle medium for the rapid oxidation of glucose and subsequent utilization of the acids produced (Holmes and Levinson, 1967). These workers also observed that during outgrowth and before microcycle sporulation of B. megaterium could be induced,

the ability to oxidize acetate had to be developed. Fluoroacetate inhibited microcycle sporulation and this inhibition could be overcome by citrate, isocitrate, glutamate, succinate, fumarate or oxaloacetate. It was concluded that , like other sporulation processes, a functioning T.C.A. cycle is probably a pre-requisite to the process.

The state during outgrowth at which sporulation can be induced has also been carefully studied (Vinter and Slepecky, 1965; Mychajlonka et al., 1975). Vinter and Slepecky (1965) studied the optimal time for the induction of microcycle sporulation, by diluting a growing culture of B. cereus, they found that spores immediately after germination or when beginning to swell could not be induced to sporulate, but that immediately after swelling, microcycle sporulation could be induced. On the other hand, the cells lyzed when the medium was diluted after the first cell division had occurred. The optimum time of induction corresponded to the time of deoxyribonucleic acid replication during outgrowth. This agrees with the finding of Dawes et al. (1971) who showed that in synchronized, rapidly growing vegetative cells of B. cereus, the ability to sporulate was a function of the time in the division cycle of the vegetative cell and limited to a period just before the completion of chromosome replication.

Mychajlonka et al. (1975) reported that microcycle sporulation ability in B. megaterium occurred prior to the onset of first cell division and declined as the cells approached that division. In addition to a requirement for the initiation of deoxyribonucleic acid replication, the cells were shown to require a certain size

before they could undergo the process. They found cells that had undergone a sphere-to-rod transition sporulated and proposed that an elongation mechanism associated with a septation site must be constructed before microcycle sporulation can occur.

Microcycle spores are "normal" in many respects. They are heat stable (though somewhat less heat resistant than the primary spores) and required heat activation for germination; they also have similar germination characteristics, dipicolinic acid content, size and shape as the primary spores (Holmes and Levinson, 1967; Mackechnie and Hanson, 1968). However, microcycle spores cannot themselves undergo another cycle of microcycle sporulation (Rodenberg et al., 1972) and they were reported to have triple the amount of deoxyribonucleic acid compared to primary spores (Holmes and Levinson, 1967).

Microcycle sporulation offers a good and simple system for the study of transformation of a primary cell into a spore in a relatively synchronous fashion. Unfortunately, the ability of Bacillus species to undergo microcycle sporulation seem to be confined to B. cereus and B. megaterium. No success was made with B. subtilis (Ramaley and Burden, 1970) and it has not been mentioned with B. stearothermophilus.

3. THE STRUCTURE OF BACTERIAL SPORES

A. CYTOLOGY

In recent years, the structure and chemistry of bacterial spores have been exclusively reviewed by many authors (Murrell, 1967; 1969; Fitz-James and Young, 1969; Tipper and Gauthier, 1972). In this section, a brief description of the chemical composition of bacterial spores will be made. This is to enable some of the spore properties observed with the present study be interpreted in the light of what is known of the chemical nature of spores.

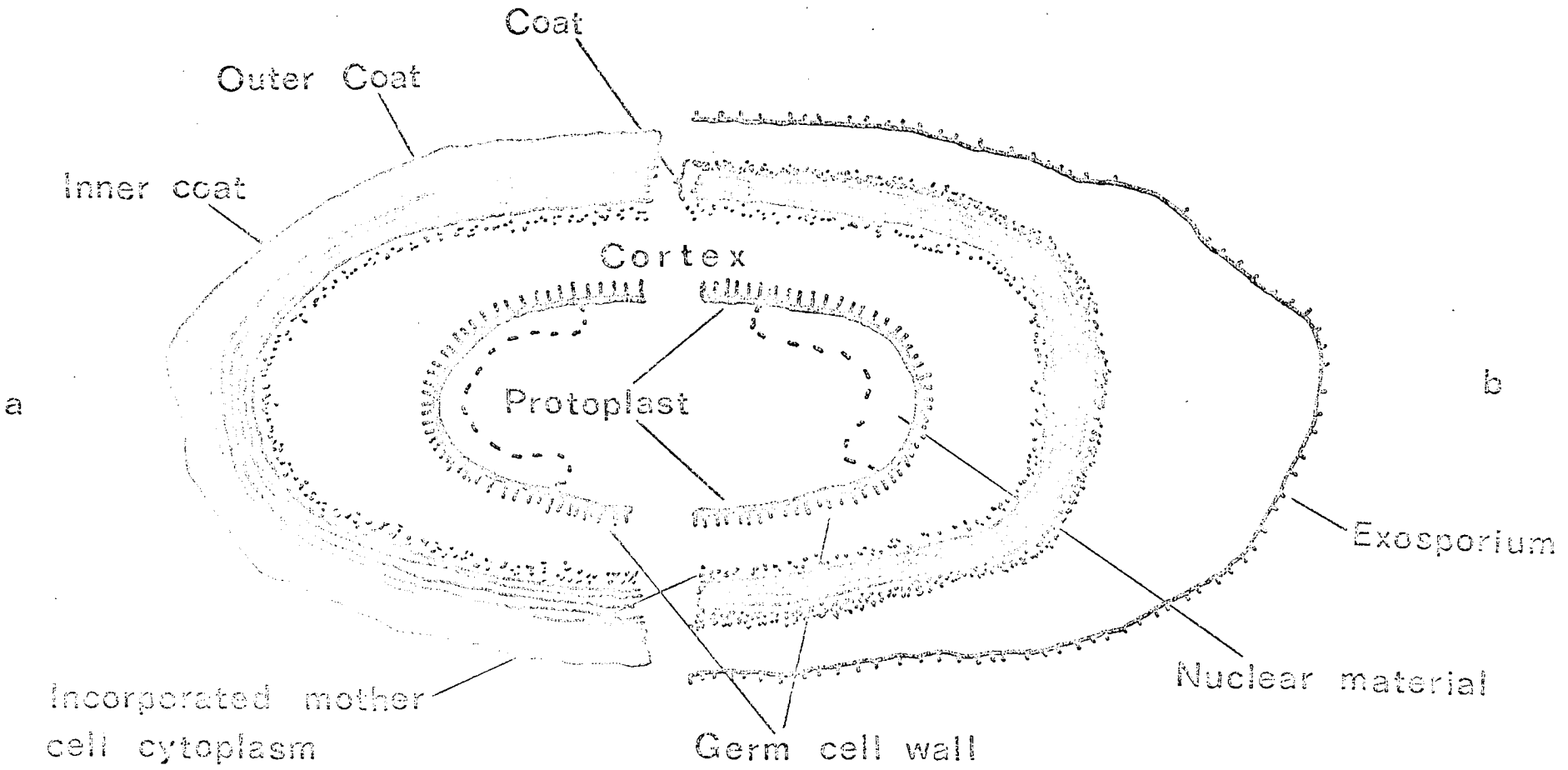
A typical spore structure is shown diagrammatically in Figure 1. It consists of a central protoplast, enclosed by the plasma membrane (or germ cell wall), around which is a thick cortex layer and multicellular coats to the outside. Incorporated mother cell cytoplasm may exists between the cortex and the coat. In B. cereus, B. cereus var anthracis (Gerhardt and Ribi, 1964), a complex ultrastructure called the exosporium lies outside the coat. Bacillus stearothermophilus spores do not possess an exosporium (Murrell and Warth, 1965).

B. GROSS CHEMICAL COMPOSITION

In general, spores contain 68 - 76% protein, about 5% carbohydrate (Table II, Murrell, 1969) and 6 - 11% ash (Table IV, 1969). Because the spore is resistant to heat and chemical treatments that denature protein of vegetative cells, there are suggestions that a class of resistant and therefore unique proteins exist in spores

FIGURE 1

Diagramatic cross-section of two structural spore types.



Type a

Spores without an exosporium
e.g. B. stearothermophilus

Type b

Spores with an exosporium
e.g. B. cereus

(Halvorson, 1965; Kornberg et al., 1968). However, about 75 - 90% of the soluble protein of spores are synthesized during sporulation (Monro, 1961; Spudich and Kornberg, 1968), this raised the question of whether the spore proteins do differ substantially from those of vegetative cells (Murrell, 1967). Several comparisons of spore and vegetative cell enzymes have indicated that spores contain many of the enzymes that occur in the vegetative cells (Murrell, 1967; Waites, 1968). Engelbrecht and Sadoff (1969) studied the purine nucleoside phosphorylase (PN-phosphorylase) from spores and vegetative cells of B. cereus. The enzyme from vegetative cells was found to be more anionic than that from the spores during gel electrophoresis in low concentration of phosphate buffer. It also has a high turnover number. The molecular weight of spore enzyme increased while that of the vegetative cells remained constant over the phosphate concentration range of 0 to 0.05 M. This shows that the enzymes from the two different sources did differ in their physical properties though they may catalyze the same enzymic reaction. Similar studies was also made with spore and vegetative cell fructose 1, 6-diphosphate aldolase from B. cereus (Sadoff et al., 1969). The enzyme from the two sources had similar heat resistance, antigenic properties, pH optima and Km values, but differed in other respects. The addition of Ca^{2+} increased the heat stability of the spore aldolase whilst decreasing the resistance of the vegetative enzyme. The enzyme also differed in electrophoretic mobility in polyacrylamide gels, in Stokes' radii and molecular weight. It seemed that the syntheses of the spore and vegetative enzymes were directed by the same genome and the vegetative type enzyme was

converted to spore type enzyme during sporulation. Sadoff et al. (1970) later showed that protease formed during sporulation would bring about the vegetative-to-spore modification of aldolase whereas non-spore proteases (trypsin, pronase) did not bring about the modification.

Setlow (1974) identified several low molecular weight basic proteins unique to the dormant spores of B. megaterium. These proteins accounted for 20% of the total protein in spores. They were not found in the germinated spores, vegetative cells or early stationary phase cells, but appeared during sporulation about 1 hr. prior to the appearance of refractile spores. These proteins also degraded rapidly during the first 20 minutes of spore germination.

Metals, particularly calcium account for most of the high ash content of spores. Dipicolinic acid is also uniquely high in all bacterial endospores and account for 5 - 15% of the dry weight and presumably occurs as calcium chelate or as dipicolinic acid-calcium-amino acid complex (Murrell, 1967).

Sulfolactic acid occurrence is unique to B. subtilis and could account for as much as 6% of the dry weight (Nelson et al., 1969). The lipid content of spores varies from about 1 - 13% of the dry weight in Bacillus species to 38% in Clostridium species (Murrell, 1969).

C. THE CHEMICAL COMPOSITION AND FUNCTION OF INDIVIDUAL SPORE COMPONENTS

(a) Exosporium

The chemical composition of the exosporium is unique. Isolated exosporia of B. cereus contained 52% protein, 20% polysaccharide, 12.5% neutral lipid, 5.5% phospholipid (all cardiolipin) and 3.8% ash. The protein of the exosporium has a low content of methionine and cysteine. They are highly resistant to proteases (Matz et al., 1970). Exosporium may have a role as additional protective layer. Its function is not indispensable as judged by its occurrence in only some Bacillus species (B. cereus and B. anthracis). It has also been implicated as having a specific role in directing coat assembly during sporulation, by compartmentalization and concentrating the coat subunits so that they do not randomly assemble elsewhere in the cell (Tipper and Gauthier, 1972).

(b) Spore Coats

Spore coats are the non-living components of spores. This is illustrated by the experiments of Fitz-James (1971) who showed that spores of B. cereus, B. subtilis and B. megaterium stripped of their coats by selective extraction were still viable and heat resistant. Technically, coat preparations are defined as the material which remains insoluble after breakage of spores and exhaustic hydrolysis with lysozyme. Analysis of such preparation of Bacillus species showed that they make up of 40 - 60% of the spore dry weight and contained up to 80% of the spore protein

(Murrell, 1967), the remainder being lipid, ash, carbohydrate and a considerable amount of phosphate. Spore coats of B. megaterium are also typically high in cysteine, glycine, lysine, aspartate, glutamic acid and non-polar amino acids (Tipper and Gauthier, 1972).

Kondo et al. (1975) found that different type of phosphorus compounds existed in the spore coats of different species of spore-formers. Phosphoryl-serine was the major phosphorus compound in the spore coat of B. subtilis, whereas phosphogalactosamine was found in the spore coat fraction of B. megaterium.

Kondo and Foster (1967) fractionated the coat preparation of Bacillus species into 3 fractions, namely an alkaline soluble fraction, a fraction solubilized upon subsequent brief sonication treatment and an insoluble, pronase-resistant fraction. The alkaline-soluble fraction would reaggregate when suspended at low pH. Its origin is believed to come from the inner coat. The sonically solubilized fraction was rich in cysteine, glycine and dicarboxylic amino acids. This fraction assembled to form paracrystalline fibrils when concentrated. It probably corresponds to the fibrillar coat layer as often revealed in freeze-etching preparation (Leadbetter and Holt, 1969). The insoluble fraction was low in cysteine but rich in lysine. It also contained considerable amounts of muramic acid and 30% ash. It was believed to originate from the outer coat. The spore coats protect the cortex from physical and enzymatic attack. B. cereus T or B. megaterium spores treated with 1% sodium dodecyl sulphate and 0.1 M. dithiothreitol removed spore coats. The treated spores retained heat resistance, refractility and all the dipicolinic acid.

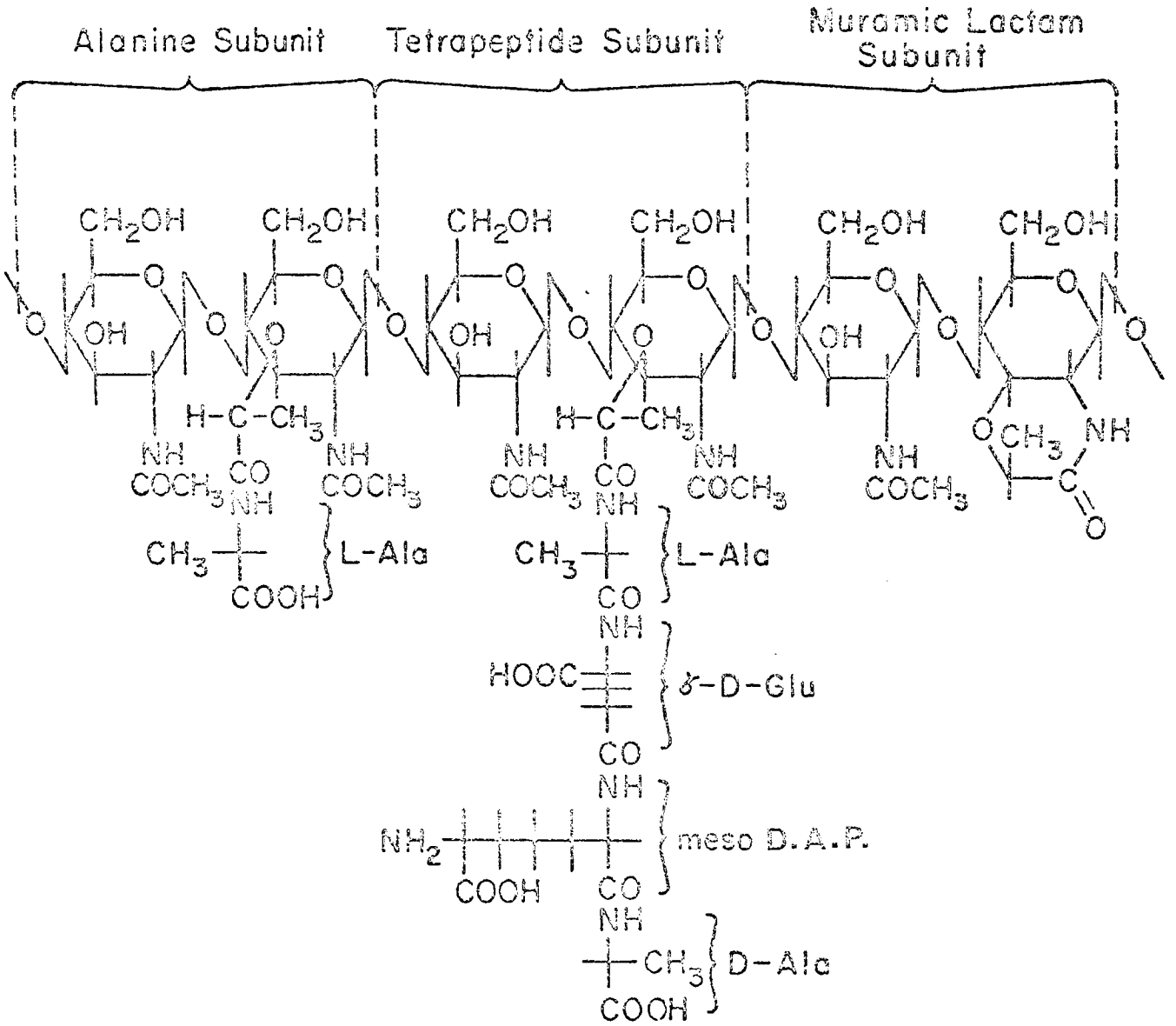
However, they clumped and frequently had damaged cortexes. Their cortexes were also rapidly hydrolyzed by enzyme (Fitz-James, 1971). The spore coats are also responsible for the resistance of spores to surfactants. Cortex-deficient B. subtilis spores with normal spore coats are not heat resistant but are resistant to octanol (Fukuda and Gilvarg, 1968).

(c) Cortex

The cortex is generally prepared by breaking spores under conditions which inhibit all autolytic activity (pH 9 - 10, plus E.D.T.A. to bind calcium). The insoluble cortical fraction was then removed by lysozyme or spore lytic enzymes. (Murrell, 1969). The cortex is composed mainly of peptidoglycan and small amount of ash. A hypothetical structure of cortical peptidoglycan is shown in Figure 2.

FIGURE 2

HYPOTHETICAL REPEATING UNITS OF SPORE CORTEX PEPTIDOGLYCAN (AFTER TIPPER AND GAUTHIER, 1972).


Alanine Subunit
Tetrapeptide Subunit
Muramic Lactam Subunit
L-Ala
L-Ala
δ-D-Glu
meso D.A.P.
D-Ala

The glycan backbone is made up of an alternating β 1-4 linked sequence of N-acetyl glucosaminyl and N-acetylmuramyl residues. In B. subtilis and B. sphaericus, about 30% of N-acetylmuramic acid residues are amide linked to the tetrapeptide L-alanyl-D-isoglutamyl- (L)- mesodiaminopimelyl- (L)-D- alanine. One in five of these tetrapeptides is cross-linked, giving an average of 6% cross-links per disaccharide unit. Another 15% of the N-acetyl muramic acid residues are substituted by a single L-alanine residue. The remaining 55% of the muramic acid residues exist as muramyl lactams (Warth and Strominger, 1969; Tipper and Gauthier, 1972). Cleveland and Gilvarg (1975) studied the degradation of peptidoglycan during spore germination by using ^{14}C-diaminopimelic acid-labelled B. megaterium spores. They found only 80% of the diaminopimelic acid were released into the medium by the spore autolytic enzymes, the remaining 20% of peptidoglycan (with greater degree of cross-linkings) were resistant to the action of enzymes. Conceivably, there are two types of peptidoglycan in the intact spores. The two types differ significantly in their per cent cross-linkings. Moreover, the percentage of cross-linking of germinated spore peptidoglycan was unaffected by exposure of the germinated spores to penicillin, thus eliminating the suggestion that transpeptidation occurred during germination.

Lewis et al. (1960) proposed that cortical peptidoglycan exists as a polyanionic gel, which shrinks considerably in the presence of divalent cations.

Spore cortex has been implicated in the maintenance of the

dehydration of the spore cytoplasm and hence heat resistance and perhaps dormancy as well. Cortexless spores of B. cereus lyzed together with the sporangia at the end of the sporulation process (Pearce and Fitz-James, 1971 a.). Addition of peptidoglycan synthesis inhibitors such as penicillin and cycloserine during the period of cortex synthesis gave rise to abnormal spores which are unstable and leak calcium-dipicolinic acid (Pearce and Fitz-James, 1971 b.).

(d) Protoplast

It is impossible to isolate and determine directly the chemical composition of the protoplast. Protoplast is defined operationally as the "soluble" fraction (10,000 x g. supernatant) after disruption of spores and removal of the insoluble integuments. This fraction accounts for 50 - 70% of the dry weight of spores. Chemical analyses of "soluble" fraction of Bacillus species showed that it contains about 9% total nitrogen, 4 - 6% amino nitrogen, 1 - 2% phosphorus, 6 - 7% sugars and 10 -26% dipicolinic acid (Murrell, 1967). This is the fraction that contains most of the free solutes, small molecules such as metabolic intermediates, amino acids, nucleotides and enzymes (Hanson et al., 1970).

(e) Other Spore Integuments

The chemical composition of other spore integuments such as the germ cell wall or the plasma membrane are little known because of the technical difficulties involved in their preparations. Germ cell walls have been suggested to be made up of peptidoglycan. It

has been suggested to protect the germinating spore from osmotic lysis and may forms the basis for subsequent vegetative cell wall synthesis (Tipper and Gauthier, 1972).

D. DISTRIBUTION OF DIPICOLINIC ACID AND CALCIUM IN SPORES

Evidence relating to the distribution of dipicolinic acid (D.P.A.) and calcium in spores is conflicting. D.P.A. was rapidly released when B. megaterium spores were abraded and heated (Rode and Foster, 1960 a.) or treated with ionic surfactants (Rode and Foster, 1960 b.). Moreover, D.P.A. and calcium were the principal compounds in the germination exudate of B. megaterium and B. subtilis when the cortex was degraded during germination (Powell and Strange, 1953). Consequently, these workers have suggested that D.P.A. and calcium are located outside the core and possibly occur in the cortex.

This suggestion fitted well with the concept of the contractile cortex put forward by Lewis et al. (1960). It was suggested that the peptidoglycan in the cortex is contracted by the presence of divalent cations presumably calcium. Hashimoto et al. (1960) observed that during sporulation of B. cereus, the synthesis of D.P.A. coincided with that of cortical material during the development of the spore.

Warth et al. (1963) and Murrell et al. (1969) made detailed chemical analyses of the various spore integuments and found that D.P.A. was present in the cortical fraction, in association with the peptidoglycan.

However, there are also evidence (both direct and indirect) showing that spore cortex does not contain D.P.A. and calcium (Hashimoto et al. , 1960; Knaysi, 1965; Murrell and Warth, 1965; Scherrer and Gerhardt, 1972; Leanz and Gilvarg, 1973). Hashimoto et al. (1960) suggested that D.P.A. is located within the spore protoplast since autoclaving of bacterial spores removed D.P.A. but not the cortex.

Knaysi (1965) employed the technique of "spodography" whereby a spore is microincinerated and the ash pattern later microscopically examined. This method assumes a calcium-D.P.A. chelate occurred in spore and that calcium will be converted into ash in situ. He provided evidence in favour of a core location of calcium and D.P.A. in B. cereus endospores.

Murrell and Warth (1965) found that spores of B. cereus T formed in the presence of cycloserine were unstable and showed little or no cortex, but they possessed normal D.P.A. content. The ability of cortexless mutants to accumulate D.P.A. was interpreted to mean that the D.P.A. location is interior to the cortex. Pearce and Fitz-James (1971 a.) pointed out that the cortex is essential for the maintenance but not for the accumulation of D.P.A. They found cortexless mutant of B. cereus showed normal D.P.A. synthesis and calcium accumulation, but both were rapidly lost to the medium following lysis of the sporangia and of spore protoplasts.

Scherrer and Gerhardt (1972) employed the method of electron probe X-ray microanalysis to examine the physical state and location of calcium in B. cereus and B. megaterium spores. This method is

of special merit since the examination is made directly on an intact spore and disruption which is known to lead to liberation and redistribution of spore components is avoided. They provided evidence to show that calcium is distributed throughout the spore similar to the distribution of carbon. There was no evidence of its being present to any great extent in the cotex, but rather that a concentration did occur within a region equivalent to the core.

Leanz and Gilvarg (1973) used the technique of beta-attenuation analysis to locate the distribution of D.P.A. It was shown earlier that beta emission from intraspore tritium-labelled compounds was partly absorbed before the particles escaped from the spore. Thus, when a label is situated deep within the spore, fewer electrons will escape and the level of radiation detected by a surrounding liquid scinter will be reduced compared with a superficially located label. Using B. megaterium cultures, they introduced various tritium-labelled markers during sporulation at different known locations within the spore. (T)-uracil introduced would mark the core nucleic acid. Similarly, (T)-α-ϵ - diaminopimelic acid would mark the spore cortex and (T)- lysine, the outer protein coat (see Section 3. C. (b)). For the unknown location of D.P.A., (T)- D.P.A. was added to sporulating cells of a B. megaterium mutant that incorporated D.P.A. into the spores. Attenuations in β-emission of 26, 18 and 3% were found for the uracil, diaminopimelic acid and lysine markers respectively. A value of 33% was found for D.P.A. marker suggesting strongly a core location of this compound.

Germaine and Murrell (1974) used the method of ultra-violet

radiation to locate D.P.A. in B. cereus spores. U.V.-irradiation of intact spores had been previously shown to cause the formation of covalent linkages between D.P.A. and its molecular neighbours via U.V.-induced free radical attachment. Intact spores containing radioactive D.P.A. were first irradiated with U.V. light and the irradiated spores were ruptured mechanically with glass beads. The distribution of the photochemically bound radioactivity among the major spore components was determined. They provided evidence to show that D.P.A. resides in the inner forespore membrane (I.F.S.M.) and spore cytoplasm. Only that portion of the D.P.A. in the vicinity of the I.F.S.M. appeared to form U.V.-induced D.P.A.-protein adducts.

E. THE NATURE OF THE CORTEX IN AN INTACT SPORE

(a) Anhydrous Core

Several indirect pieces of evidence have shown that the spore interior is low in water. Spores have a low rate of metabolism (Crook, 1952) and high density (McIntosh and Selbie, 1937). Murrell and Scott (1966) found that if the water activity (a_w) at which spores were heated was lowered, the spore heat resistance was increased. This increase was greatest for those spores which are normally heat sensitive because they have high a_w values. Thus, when Cl. botulinum Type E spores were equilibrated at a_w values less than 0.5, they became as resistant as the spores of B. stearothermophilus whereas at very moist conditions (a_w = 1.0), spores of Bacillus stearothermophilus were still 50,000 times as resistant as those of Cl. botulinum. They concluded that low a_w in Cl. botulinum spores

gave rise to a situation similar to those of B. stearothermophilus, whereas high a_w had no effect on high resistant spores because these spores normally maintained a low a_w values.

Fitz-James (1971) developed a method for producing spore protoplasts by digestion of coatless spores of Bacillus species with lysozyme. In the presence of calcium and absence of magnesium, structures were formed which he called "semihydrate" and which were apparently spore protoplasts. These structures were partly refractile, indicating that the protoplast was maintained in a dehydrating state even after cortical digestion. He found that on the removal of calcium and the introduction of magnesium, a process of hydration of protoplast could be observed in electron microscopy.

Wyatt (1975) employed the technique of differential light scattering with single spores of Bacillus sphaericus and Clostridium filamentosum and provided evidence that showed the core is devoid of any free water. He proposed that D.P.A. acts as filler material in the spore core to prevent any free water penetration.

Though the spore core may be fairly anhydrous, there are evidence that showed the spore structure as a whole is freely permeable to glucose, water and small solutes which enter into the spore passively and to some extent governed by the molecular weight, charge and lipid insolubility (Black and Gerhardt, 1961 a.; Black and Gerhardt, 1961 b.; Black and Gerhardt, 1962a). Black and Gerhardt (1961 a.) employed the method developed by Conway and Downey (1950) to assess the distribution of glucose uptake into B. cereus spores.

In this method, pellet of clean spores (phase bright) obtained after centrifugation at specified speed, was equilibrated with a solution of test solute of known strength. The spores were then repacked by centrifugation at specified speed. The extent of solute uptake was assessed by the increase in the pellet weight (or decrease in the concentration of glucose in supernatant, assessed gravimetrically, isotopically, carbon combustion or anthrone analysis) and applying some derived equation. Correction for cell leakage of spore material (e.g. dipicolinic acid) was also made. Packed spores of B. cereus had been reported to be permeated by glucose up to 40% of their weight (Black and Gerhardt, 1961 a.) and 66.6 to 86.0% by tritium-labelled water (Black and Gerhardt, 1962a). The conclusion that passive diffusion principally accounts for the uptake is shown by the fact that uptake was not influenced by temperature changes; the equilibration time (after 15 minutes) did not significantly change the extent of uptake and the glucose taken up by a spore pack could be completely recovered by washing the spores in water. Moreover, the uptake was not influenced by H^+ concentration and the total solute uptake contained in a spore pack was proportional to the solute added. The extent of spore damage resulting from spore packing was not determined. The spores after the experiment were reported phase-dark and did not produced colonies when plated. It seems likely that structural damage of spores during the process could account for some of the uptake observed.

(b) Theory Of The Contractile Cortex

Lewis et al. (1960) proposed that the cortex is contracted.

They reckoned that a truly water impermeable coat is inconsistent with the known permeabilities of organic materials and suggested that a low water in the spore protoplast could arise through compressive contraction of the cortex during sporulation.

Since then, several workers have provided evidence in-support of the concept (Hitchins and Gould, 1964; Marquis, 1968; Ou and Marquis, 1970). Hitchins and Gould (1964) showed that the isolated spore core of B. subtilis with residual cortex attached became contracted and increased in refractility as the pH of the medium was lowered or as the multivalent cation level was increased. Ou and Marquis (1970) observed that peptidoglycan in the isolated vegetative cell walls of Staphylococcus aureus and Micrococcus lysodeikticus contracted when reacted with salts such as $CaCl_2$, $MgCl_2$, KCl or NaCl. Caslavska et al. (1970) reported that a variety of basic compounds (polymyxin B, polylysin etc.) when added to germinated spores of B. cereus caused increase in refractility, presumably by cross-linking and contracting residual peptidoglycan fragments in the spore.

(c) Theory Of The Expanded Cortex

The theory of the contractile cortex predicts that the spores contain a high level of peptidoglycan compared with the vegetative cells. The peptidoglycan is highly contracted and compressed, thus occupying only a small volume. This prediction is incompatible with the finding of Murrell (1967) who showed that the amount of peptidoglycan in spores was not significantly greater than that of the vegetative cells. Moreover, Gould and Dring (1974) showed that the volume

occupied by the spore cortex of Bacillus species was exceptionally large (30 - 60%), compared with 23% in the cell wall of the corresponding vegetative cells.

This led Gould and Dring (1974) to propose that the cortex is expanded, the expanded electronegative peptidoglycan necessitates the absence of neutralizing cation or other basic molecules in the cortical region. The hypothesis was substantiated by experiments in which spores were heated in high concentrations of cross-linking divalent and trivalent cations, but not monovalent cations, became heat-sensitive. This sensitization phenomenon occurred only with coat defective mutant, or spores in which the coats had been chemically modified by reacting with urea-mercaptoethanol, urea-thioglycollic acid or urea-dithiothreitol. The expanded cortex theory predicts that the cortex contains a high water content and it is the water in this structure that provides the expansion. The expansive pressure generated is believed to lower the water content of the core. The cortex thus acts as a specialised osmoregulatory organelle. Changes in the environment which reversibly affect the osmotic properties of cortex alter the heat resistance of spores.

Dring and Gould (1975) found that the heat resistance of ungerminated B. cereus spores depended upon the dehydration of the central core. During germination, rehydration of the core occurred accompanied by greatly decrease resistance to heat. However, heat resistance could be completely regained by reimposing dehydration in the core by osmosis, e.g. by resuspending the germinated spores in sufficiently high concentrations of non-permeant solute like sucrose, thus

recreating conditions approximating those in the ungerminated spore.

Warth and Strominger (1969; 1971) found that the amino sugar backbone of peptidoglycan of B. subtilis spores was more loosely cross-linked with its peptide side chains than was the case of vegetative cell peptidoglycan. This loosely cross-linked structure is therefore more expanded.

The expanded cortex theory is also compatible with the increasing evidence, calcium-D.P.A. is present in the protoplast and not in the cortex (see Section 3. D.). Whether or not the cortical region in the spore is as hydrated as the theory suggests remained to be tested further.

4. THE IMPORTANCE OF DIPICOLINIC ACID AND CATION CONTENTS ON SPORE PROPERTIES

A. THE IMPORTANCE OF D.P.A. CONTENT ON SPORE PROPERTIES

Dipicolinic acid occurrence is unique to bacterial spores. In most Bacillus species, the content of D.P.A. ranges from 5 to 15% of dry weight of spores (Murrell, 1967).

Hashimoto et al. (1960) studied the synthesis of D.P.A. during sporulation of B. cereus and found that the synthesis of D.P.A. paralleled and preceded the development of thermoresistant spores. It is this close relationship between D.P.A. accumulation and the formation of thermoresistant spores that led many workers (Young, 1959; Church and Halvorson, 1959; Hashimoto et al., 1960) to suggest that the D.P.A. content of spores is positively correlated with the heat resistance of spores. Young (1959) suggested that D.P.A. and calcium act together in stabilizing essential protein and nucleic acids. She found chromatography of spore extracts of B. megaterium produced a D.P.A. spot with tailing which was found to be due to the presence of six amino acids. Church and Halvorson (1959) prepared spores of B. cereus T with varying D.P.A. contents by manipulating the concentration of DL-phenylalanine which is inhibitory to D.P.A. synthesis, in the medium. They found thermal inactivation of these spores at 80°C, showed a biphasic dependent on the D.P.A. content and concluded that D.P.A. protects the spore against heat inactivation in at least two sites. Zytokoviz and Halvorson (1972) showed that D.P.A.-minus mutants of B. cereus T and B. megaterium 899 were extremely heat sensitive whereas the corresponding wild types

were heat resistant.

Several workers (Bryne et al., 1960; Walker et al., 1961; Grecz and Tang, 1970) on the other hand, could not find any correlation between heat resistance and D.P.A. content in spores. Walker et al. (1961) analyzed the nitrogen, carbohydrates, phosphorus, calcium, magnesium and dipicolinic acid contents in a total of 17 strains of 4 species (B. polymyxa, B. megaterium, B. cereus and B. subtilis). Heat survival curves were determined in each strain, which were then ranked in the order of heat resistance. They found no correlation between the nitrogen, carbohydrates, phosphorus, dipicolinic acid or Ca/D.P.A. molar ratio and the heat resistance of spores. However, heat resistance was increased as the molar ratio of Ca/Mg or D.P.A./Mg was increased. It should be pointed out that these workers used spores prepared by washing from the agar surfaces with acidified water (pH 2.0 to 2.5). The spores were then subsequently washed six to eight times by alternate suspension and centrifugation in acidified water. The effect of four times acidic washing and eight times acidic washing was compared with respect to the content of nitrogen, phosphorus, D.P.A. and carbohydrates in spores. It was reported that the number of acidic washings have no effect on the content of these constituents. However, no comparison was made between non-acidic and acidic washing with respect to spore composition and properties. It seems likely that some loosely bound D.P.A. and cations could have been removed after four acidic washings (see Brown and Melling, 1967; Crosby et al., 1971) and the comparison made between the fourth and eighth acidic washing represents the comparison

between the contents of much tightly bound spore components. In this respect, the conclusion obtained by Walker et al. (1961) may well represent the relationship between tightly bound spore components and heat resistance. The contribution of spore components lost during early acidic washings to spore heat resistance can never be assessed.

Bryne et al. (1960) studied the heat resistance of Clostridium roseum spores grown in media supplemented with varying levels of L-alanine. They found the levels of D.P.A. in spores were altered by the presence of different concentrations of L-alanine in the medium. Increasing the concentration of L-alanine resulted in a lowering of spore D.P.A. content and accompanied unexpectectedly by an increase in the spore heat resistance.

Grecz and Tang (1970) studied spores of five strains of Cl. botulinum with D.P.A. content ranging from 7.4 to 13.4% of the dry weight and found no correlation between the heat resistance and the D.P.A. content of spores.

Hanson et al (1972) isolated a revertant of a D.P.A.-negative B. cereus T mutant. The spores of this bacterium were D.P.A.-negative and yet as fully heat resistant as the D.P.A.-positive wild type spores. These spores contained one-tenth to one-twentieth of the wild type calcium content and the heat resistance was not well maintained. Thermoresistance was lost following lyophilization or two weeks storage at $4^{\circ}C$ or after cleaning in a two-phase polyethylene glycol phosphate buffer. These spores were also slow

to germinate under conditions that initiated the germination of wild type spores. This led Hanson et al. (1972) to suggest that D.P.A. functions to stabilize established heat resistance and that its major functions are probably in the maintenance of dormancy and the initiation of germination. This suggestion is in contrast with the finding of Keynan et al. (1961); they showed that spores of B. cereus T grown so as to contain low levels of D.P.A., did not require heat shock for germination, whereas heat shock was required for the germination of spores with high endogenous D.P.A. On the other hand, Halvorson and Swanson (1969) found that mutant spores of B. cereus T, devoid of D.P.A. required D.P.A. for germination.

D.P.A. absorbs strongly in the U.V. region (Grecz et al., 1973). Berg and Grecz (1974) studied the mutant of B. cereus T that produced spores devoid of D.P.A. They found that the U.V. and γ radiation resistance of these spores were statistically reduced. This observation seems to contrast that of Black et al. (1960) and Vinter and Vechet (1964). These workers reported that the spores with low D.P.A. content had the same or even greater radiation resistance than "normal" spores. Rowley and Newcomb (1964) reported that radiation resistance in spores appeared before D.P.A. formation during sporulation of B. subtilis and so could not have been responsible for the radiation resistance observed.

B. THE IMPORTANCE OF CATION CONTENT ON SPORE PROPERTIES

Spores contain a wide variety of inorganic ions, the major ones include Ca^{2+}, K^{+}, PO_4^{3-}, Mg^{2+} and Mn^{2+}. Calcium accounts for as much as 2 - 3% of the dry weight of Bacillus spores (Thomas, 1964;

Murrell, 1967). The notion that the metal ion content of spores is important for heat resistance stems from the observation that the conditions of sporulation, particularly the levels of ions in the medium affects the heat resistance and the ionic content of the resulting spores (Slepecky and Foster, 1959; Levinson and Hyatt, 1964; Aoki and Slepecky, 1973).

Levinson and Hyatt (1964) reported that the heat resistance of B. megaterium spores was related to the molar ratio of calcium to D.P.A. This ratio increased with the increase in the heat resistance of spores. This report, however, was based on the determination in each of the four species studied and the heat resistance was simply measured as percentage survivors after exposure to 90°C for 20 minutes. The possibility that different shaped survivor curves might be exhibited by different species was not considered.

Murrell and Warth (1965) analysed spores of Bacillus species with a 700-fold range in heat resistance. They found the calcium content of spores was positively correlated with their heat resistances. The Mg/Ca molar ratio decreased significantly with corresponding increase in the heat resistance of spores. These workers concluded that the calcium content in spores is essential for the attainment of heat resistance, but the role of magnesium is not known. It is unlikely to displace calcium since spores with high magnesium content did not have lower Ca/D.P.A. ratio. On the other hand, Slepecky and Foster (1959) showed that when spores were grown in medium containing fixed amount of calcium (1.8 µg/ml.) together with low and high levels of other divalent cations (Zn^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+} and Mn^{2+}). The resultant

calcium level in spores was decreased in the presence of high concentration of other ions which themselves exhibited enhanced levels in the spores.

Rowley and Levinson (1967) found spores of B. megaterium treated with thioglycollate (0.4 M., pH 2.6) for 5 minutes at 30°C retained D.P.A. but lost their heat resistance and germinability, which could be restored by supplying exogenous cations.

Hanson et al (1972) isolated a revertant of a D.P.A.-negative B. cereus T mutant. Spores were prepared from this bacterium in medium free of added calcium. The levels of calcium and manganese in these spores were low, but the spores were as fully heat resistant as the wild type spores. They observed that the heat resistance and germinability of these spores were lost upon storage. Since increasing evidence point to the possible core location of D.P.A. and calcium (see Section 3. D.), this led Gould and Dring (1975) to propose a new role for calcium-D.P.A. in spores. They proposed that calcium-D.P.A. acts as a buffer and the calcium in intact spore is lost slowly by leakage during storage, the calcium loss is quickly and very efficiently replaced by the dissociation of calcium-D.P.A. complex located in the core. Mutants lacking D.P.A. were said to have a low internal reserve of calcium, sufficient only to satisfy the requirement for the maintenance of dormancy and heat resistance over a short period of time, thus dormancy and heat resistance of these spores are not well maintained during prolonged storage.

On the basis of U.V. spectra of dry spores embedded in KBr,

Bailey et al. (1965) reported the presence of manganese-D.P.A. chelate in spores. Aoki and Slepecky (1973) prepared spores of B. fastidiosus with and without manganese in the medium. The manganese content of these spores was positively correlated with the concentration of manganese in the medium. There was also positive correlation between the manganese concentration of the medium and the thermoresistance of spores. Spores prepared from medium with added manganese germinated only after heat activation and the effectiveness of this heat activation was directly dependent on the concentration of manganese in the growth medium.

5. THE KINETICS OF DIPICOLINIC ACID AND CATION RELEASE IN RELATION TO SPORE PROPERTIES

The Kinetics of D.P.A. and cation release from spores following heat treatment have been implicated in at least two areas of spore properties, namely the maintenance of dormant state and heat resistance.

Harrell and Mantini (1957) found that following heat shock treatment at 65°C in pH 7.2 phosphate buffer, spores of B. cereus var terminalis oxidized glucose. The degree of oxidation increased with the time of heating and during this treatment, the amount of D.P.A. release increased from 2% of the total at zero time to 12.5% of the total after 60 minutes. The initiation of glucose oxidation necessitated the activation of enzymes which have been known to become active during germination. A hypothesis was therefore posed that germination ensues only after the endogenous D.P.A. content is reduced to a critical level.

Keynan et al. (1961) determined the percentage of D.P.A. released after sufficient heat shock to permit optimal germination for a variety of spore stock with varying D.P.A. levels. They found no correlation between the amount of D.P.A. released and germination. However, optimal germination occurred with about a 10% loss of D.P.A. They proposed that several reservoirs of D.P.A. probably exist in the spore and only a minor fraction of which control dormancy.

Brown and Melling (1967; 1973) found that the rate of release of D.P.A. from B. stearothermophilus spores and the breaking of

dormancy of these spores were both functions of time, temperature and pH. Thus, lowered pH activated as did raising the temperature. They showed that the apparent activation energies for D.P.A. release and the rate of dormancy breakage were similar and proposed that the nature of the D.P.A. bond, rather than the content of D.P.A. is a determining factor for the maintenance of dormancy and reisitance.

Lund (1958) found that the percentage of total D.P.A. released after heat treatment was correlated with the heat resistance of spores. This was later confirmed by Walker and Matches (1965), they showed that the most heat resistant spores released the least amount of D.P.A. Grecz and Tang (1970) ranked in order the degree of heat resistance of 5 strains of Cl. botulinum and showed them to be correlated with the rate of release of D.P.A. during heat treatment. No relationship existed between the total D.P.A. content and heat resistance. To explain the loss of viability on heating without any apparent D.P.A. loss, they suggested that D.P.A. release may involve at least two distinct sequential steps: (1) breaking of D.P.A. complexes from attachment sites; and (2) leakage of D.P.A. complexes through the spore membranes and coats. Steps (1) may result in loss of viability, but only step (2) would permit detection of D.P.A.

Levinson and Hyatt (1971) showed that the loss of viability of B. megaterium spores when heated between 75° to 85°C was more rapid than corresponding loss of D.P.A. and germinability, and although these events could not be correlated by use of simple kinetic plots, they had similar activation energies (80 - 90 k cal./mole).

Hodges and Brown (1975) studied the effect of growth medium on the rate of release of D.P.A. and cation from B. megaterium spores. Spores prepared from simple salt media and nutrient agar were found to possess different heat resistances which could be correlated with the rate of D.P.A. and cation (magnesium and calcium) release. This relationship was apparently unaffected by the nature of growth medium or the concentration of medium manganese.

6. ION EXCHANGE AND SPORE PROPERTIES

Bacterial spores exhibit a considerable capacity for ion exchange (Halvorson and Howitt, 1961; Alderton and Snell, 1963). Halvorson and Howitt (1961) prepared spores of *B. cereus* T from medium containing ^{45}Ca and then placed the radioactive spores in a medium containing 1.45 mM $CaCl_2$. They reported that nearly 35% of the initial metals were being exchanged by the non-radioactive calcium in $CaCl_2$.

Alderton and Snell (1963) showed that lyophilized spores of *B. megaterium* held at 25°C in pH 4 for 4 to 5 hours were acid-stripped and "H"- spores were produced. They found "H"- spores retained their viability but became heat sensitive. Heat resistance was restored when acid-stripped spores were exposed to calcium hydroxide solution at pH 9.5. This finding is important because the level and nature of metal ions in spores can be controlled without modifying the ionic composition of the sporulation medium. Spores can be acid-stripped and reloaded with the metal ions of choice and the properties determined.

In quantitative terms, the amount of calcium removed from spores after the acid-stripping process was only 5% of total calcium content. Of this 5%, it was found that only a portion was correlated with the spores' thermal resistance (Gould and Dring, 1975).

The process of restoration of metal ions upon reloading was studied by Alderton *et al.* (1964) using *B. megaterium* spores. They showed that when "H"- spores were rapidly exposed to lethal temperatures in 20 mM calcium acetate buffer, logarithmic order of death was

replaced by decelerating death rate. Likewise, "H"- spores exposed to calcium buffer at lower than lethal temperature or through the increasing of warm-up time at the lethal temperature, the initial slope of kill was progressively reduced, indicating that re-adjustment within the spore took place in relation to its calcium complement. It was also established that the restoration of calcium content occurred in two stages; first, a rapid uptake not correlated with the re-acquisition of heat resistance took place. This was followed by a slow phase of calcium uptake which correlated with the restoration of heat resistance in spores. The second phase of uptake was both temperature and pH dependent.

The effects of acid-stripping on spore dormancy and germination were studied by several workers (Lewis et al., 1965; Rode and Foster, 1966). Lewis et al. (1965) found spores of B. stearothermophilus were activated by exposure to an acid medium at pH 1.5 (cf. Brown and Melling, 1973). Dormancy was reversibly restored by exposure to an alkaline solution of calcium ions.

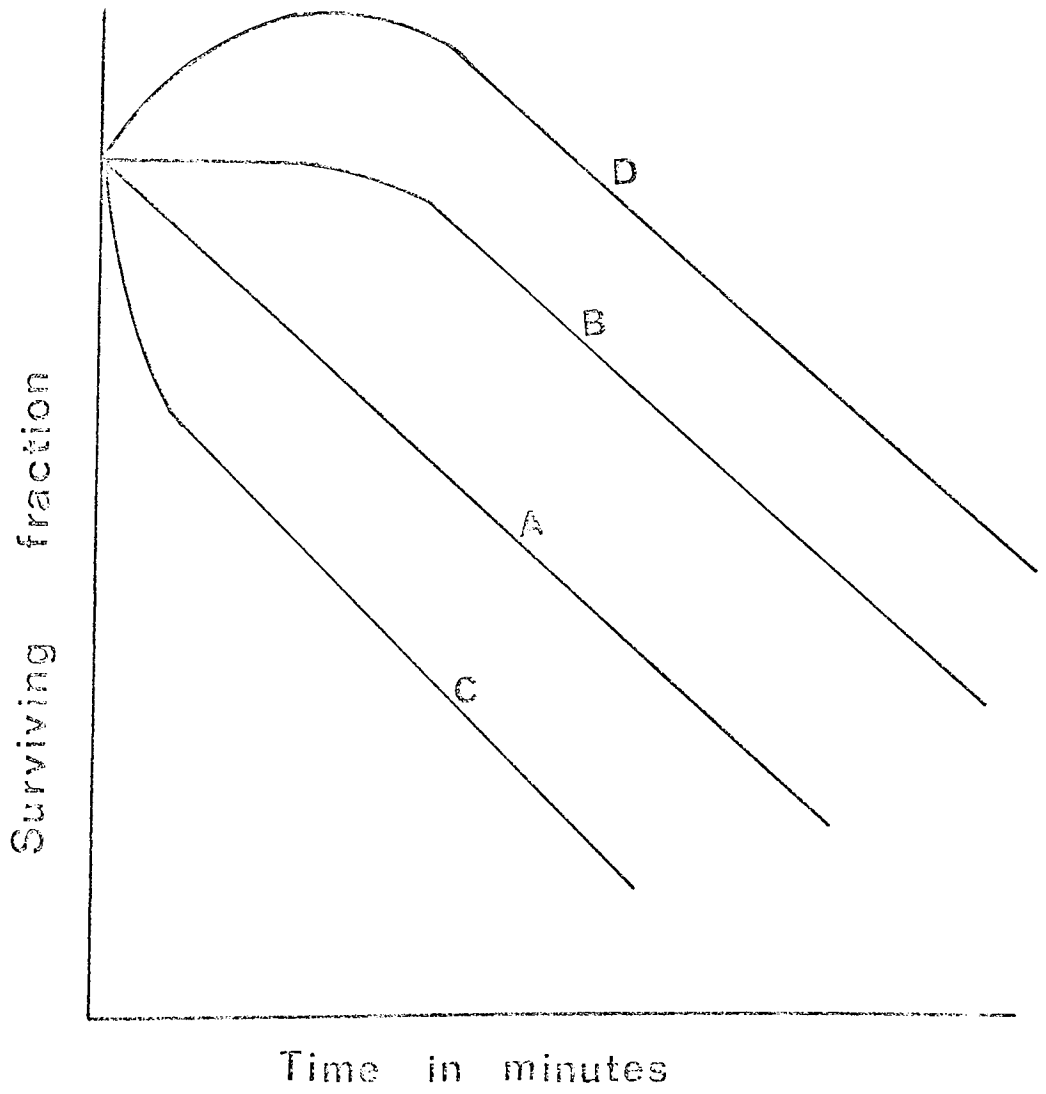
Rode and Foster (1966) found unstripped spores of B. megaterium germinated quickly in a solution of L-alanine and inosine whilst the acid stripped spores did not germinate in such a system unless a strong electrolyte was present. When acid-stripped spores were reloaded with calcium, they germinated efficiently in the same system without the presence of the strong electrolyte. Of the several bivalent cations tested, only strontium and barium could substitute for calcium requirement in conditioning spores for subsequent germination in the absence of a strong electrolyte.

Splittstoesser and Parkas (1966) found that heat activation of B. popilliae spores was conditioned by the ionic composition of the suspending medium. Maximum rate of heat activation was achieved in calcium solution at pH 7 or in tris(hydroxymethyl) amino-methane buffer at pH 9 to 10. The activation rate was reduced when the medium contained K^+ or H^+ but not when it contained Na^+, Ce^+ or Li^+ implicating that K^+ could somehow compete with Ca^{2+} for some active sites.

7. THE EFFECT OF HEAT ON BACTERIAL SPORES

When bacterial spores are exposed to lethal temperatures and the fraction of survivors is plotted against time, various types of curves may be obtained (Figure 3).

FIGURE 3



Curve A is compatible with a constant fraction of spores being killed per unit time. Simple exponential kill of this type have been shown with B. megaterium spore suspensions (Hodges and Brown, 1975). Deviation from this straight line are constantly being reported and curves of either increasing death rate (concave down, Curve B) or of decreasing death rate (concave upwards, Curve C) have been shown (Anand, 1961). In addition, curves of an initial increase in colony counts, caused by heat activation followed by an exponential rate of death (Curve D) have been reported (Shull and Ernst, 1962). Reynolds and Lichenstein (1952) reported three phases of death with PA 3679 spores. An initial phase, during which an accelerated rate of death took place, when about 50% or more of the spores became non-viable, an exponential phase and finally a decreasing rate of death as the last few spores were inactivated. Ridgeway (1958) found similar triphasic responses with B. subtilis.

Several explanations of deviation from strictly exponential death have been suggested. These include the presence of two or more discrete discontinuous populations of different resistances (Vas, 1970; Levinson and Hyatt, 1971); to the acquisition of heat resistance during the initial portion of the heating period (Alderton et al., 1964; Han et al., 1976); to the requirement of heat activation of spores (Shull and Ernst, 1962); to multitarget kinetics (Moats, 1971) and to the presence of a few highly resistant spores (Ridgeway, 1958).

By treating heat resistance as a continuously distributed function, Levinson and Hyatt (1971) made probability plots of

percentage survivor as a function of log time, of aqueous suspension of B. megaterium spores, heated at temperature of 75° to 85°C. Two straight lines intersecting at survival level of 1 - 6% and occurred at time ranging from 8 minutes for spores heated at 85°C to 310 minutes when heated at 75°C. Probit-intersects for D.P.A. release and loss in germinability occurred at the same time as for survival, but at much higher levels of retention. They concluded that there were two sub-populations in the suspension, both log-normally distributed but with different mechanisms of kill. 94 to 99% of the spores were killed via injury to the cell-division process but retained germinability; the remaining sub-population (1 - 6%) was non-viable because of the loss of ability to germinate.

Han et al (1976) developed mathematical models to explain concave survival curves on semi-log paper. They reported that concave survival curve caused by innate heterogeneity of suspension was parabolic whilst that caused by acquired heat resistance during heating was exponential. The models were applied to published survival curves of B. coagulan spores (Frank and Campbell, 1957) and B. cereus spores (Han et al., 1971). For the cases studied, the cause of curvilinearity was attributed to the acquisition of heat resistance rather than innate heterogeneity of the spore population. Also, the rate of development of resistance was found to be faster with B. coagulans spores than those of B. cereus spores.

A. HEAT INACTIVATION

The enormous commercial importance of the thermal death of bacteria has stimulated a considerable amount of work in this area to make it by far the best studied aspect of the effect of temperature on bacteria. Since practical studies are directed toward eliminating the most heat-resistant form that might be present in the material to be sterilized, the thermal death of bacterial endospores has received considerably more attention than the destruction of vegetative cells (Bigelow and Esty, 1920; Kelsey, 1958).

The rate of kill of spores is undoubtedly determined by the resistance of the organism. The various factors that have been reported to influence the destruction of spores include:

(1) inherent resistance of the species (Murrell and Warth, 1965)

(2) environmental influence active during the growth and formation of spores (Lechnowich and Ordal, 1962. See Section 2. C.)

(3) environmental influence active during the time of heating of spores (Levinson and Hyatt, 1960; Cook and Gilbert, 1965).

Inherent resistance varies not only between species, but also between different strains of the same species. Murrell and Warth (1965) quoted D value, which defines the time required in minutes to destroy 90% of the population as 2.1 minutes at 100°C for B. megaterium spores and 714 minutes for B. stearothermophilus spores.

Lechowich and Ordal (1962) found that the temperature of

cultivation influenced the shape of survival curve. B. subtilis spores produced at 30°C showed exponential death when heated at 98.5°C in M/40 phosphate buffer, whereas spores produced at 45°C showed an initial shoulder, followed by exponential death.

Levinson and Hyatt (1960) reported that B. megaterium spores were more resistant when heated in phosphate buffer than when heated in cacodylate or water. Other substances that are known to influence the heat resistance of spores when included in the heating menstrum include yeast cells (Cook and Gilbert, 1965); antibacterial agents (Denny et al., 1961).

The effect of nisin on heat resistance may be more apparent than real. Thorpe (1960) found the antibiotic adsorbed onto B. stearothermophilus spores and that, unless removed by means of trypsin, the adsorbed antibiotic carried over into the sub-culture medium prevented colony formation.

Claims that sodium chloride protects spores against heat damage (Esty and Meyer, 1922) were contradicted by the report of a progressive increase in the death rate of B. coagulans spores with increasing concentrations of sodium chloride in tomato juice (Anderson et al., 1950). Roberts et al. (1966) were unable to detect any effect of sodium chloride on resistance when used in concentrations of interest to the food industry.

Spores surviving heating have been reported to be more exacting in their growth requirements than are unheated spores (Currans and Evans, 1937; Nelson, 1943). Consequently, the nature and condition

of recovery affect the number of survivors counted. Duncan et al. (1972) reported experiments in which spores of Cl. perfringens were shown to be inactivated by heat if enumerated using conventional media, and yet appeared still viable if plated in media containing lysozyme. It seems likely that heat normally kills these spores by inactivating enzymes necessary for the initiation of germination, whilst leaving the protoplast viable but trapped within the spore. The care needed in interpreting heat inactivation data of bacterial spores was recently highlighted by finding of Busta and Adams (1972). They found that the sensitivity of B. subtilis A spores to activation and inactivation by heat differed according to the system chosen to initiate germination, suggesting that heat could damage one germination system more than another.

(a) The Mechanism Of Heat Inactivation

The mechanism whereby bacterial spores are killed on exposure to moist heat is unknown. Several theories have been proposed. Savage (1959) suggested that it is the toxic properties of water at high temperature which hydrolyze protein and denature them and that superheated steam is an inferior sterilizing agent because it does not allow the water content of the spore to increase. Amaha and Sakaguchi (1957) determined the activation energy and thermodynamic functions of death of B. natto, B. megaterium and Bacillus mycoides spores and found that the activation energy and entropy of activation were of the same order and magnitude with those of the heat denaturation of proteins such as hemoglobin, trypsin and lipase. The energy of activation was found to be 50 to 77 k cal./mol. while

the entropy of activation was 66 to 140 K cal./mol./deg. They concluded that the death of spores by heat is caused by the denaturation of some protein molecules in spores.

The death of B. stearothermophilus spores by moist heat was studied by Anderson and Friesen (1974). Two mechanisms of inactivation were reported. Below 100°C, the reaction had a free energy of activation of about 25 K cal./mol. and low entropy of activation (30 cal./mol./deg.) and so involved very few relatively strong bonds. They suggested that modification of nucleic acid molecules probably took place at this temperature. Above 105°C, the inactivation reaction had a similar free energy of activation, but much higher entropy of activation (about 150 cal./mol./deg.). The rupture of many weak bonds, probably hydrogen bonds associated with protein was suggested at this higher temperature.

B. HEAT ACTIVATION

Curran and Evans (1944, 1945) were the first workers to demonstrate systematically that sublethal heat (62 to 95°C) treatment could led to an increase in the subsequent viable counts of spore suspension. They called this phenomenon heat activation. This term was later used to describe heat treatment of spore suspension that resulted in a decrease in the lag time or an increase in the germination rate (O'Conner and Halvorson, 1961).

The temperature requirement for heat activation varies not only between species but also between different strains of the same species. Currans and Evans (1945) found that a few minutes at 60°C activated

B. megaterium spores, whereas B. stearothermophilus and other thermotolerant spores required 105° - 115°C for optimal activation. Finley and Fields (1961) showed that heat treatment of 115°C for 3 minutes was required for the maximal activation of a strain of Bacillus stearothermophilus 1518, whereas strain M required heat treatment of 110°C for 9 to 15 minutes for maximal activation.

Powell and Hunter (1955) demonstrated the presence of a "critical temperature" and "critical time" of heat activation in B. megaterium spores. These spores were heat activated at temperature of 45 - 78°C. Optimal activation was conditioned by both the temperature and duration of exposure, a very long heating period would be needed for activation at low temperatures. They found that for complete germination of B. megaterium spores at 45°C, 18 hours of exposure were needed. When the spores were held for same time at 44°C, practically no activation could be observed, showing that changes in heating time necessary to achieve maximal activation at a temperature were not linear. The existence of a "critical temperature" (when heating for a constant time) and a "critical time" (when heating at a constant temperature) was suggested and this was thought to be reflected by a sudden and abrupt rate of germination.

The exact time and temperature of activation depend to a great extent on the composition of the medium from which spores have been prepared and the age of the suspension (Keynan et al., 1961; Levinson and Hyatt, 1964). Keynan et al. (1961) prepared B. cereus var terminalis spores containing different concentrations of D.P.A. by manipulating the concentration of phenylalanine in the medium. They

found positive correlation between the hours required for activation at 65°C and the intrasporal D.P.A. concentration.

Heat activation is also determined by the conditions of medium in which spores are suspended. Powell and Hunter (1955) showed the critical importance of the presence of water during heating of B. megaterium spores. Keynan et al. (1965) reported that some salts (NaCl, KCl, LiCl and chlorides of Mn^{2+}, Mg^{2+} and Ca^{2+}) which had been shown to inhibit the heat activation of B. cereus T spores, when removed after activation by washing. These spores behaved activated.

Generally, heat activation in bacterial spores is "temporary" (Powell, 1950; Church and Halvorson, 1957). Powell (1950) pointed out that this temporary state is different from the process of ageing which is irreversible and permanent. Keynan et al (1964) studied the influence of temperature on the de-activation of fully activated spores of B. cereus during storage. They found de-activation was completed within 72 hours at 28°C. No de-activation occurred at - 20°C.

Several workers have proposed theories explaining the mechanism of heat activation (Franklin and Bradley, 1957; Keynan et al., 1964). Keynan et al. (1964) suggested that heat activation probably involved changes in a protein responsible for the maintenance of the dormant state by reducing the disulphide linkages which stabilize the protein in a specific configuration, since heat activation of spores could be imitated by reducing agents like mercaptoethanol or thioglycollate. Franklin and Bradley (1957) found heat-activated spores of Bacillus

stearothermophilus had cracked spore coats and suggested a role of spore coat in the maintenance of dormancy. Moberly et al. (1966) demonstrated morphological and structural changes in the activated spores. They found spores of B. anthracis after heating for 15 minutes at 65°C became more mottled and the cytoplasm was less granular and more opaque than in the dormant spore.

Several workers have demonstrated that heat activation alters the germination requirement of spores (Powell and Hunter, 1955; Levinson, 1961). Powell and Hunter (1955) observed that freshly harvested spores of B. cereus required either inosine or a mixture of alanine, tyrosine and adenosine for optimal germination whereas a short heat treatment, adenosine alone stimulated rapid and complete germination. Levinson (1961) found heat activated and unheated B. megaterium spores possessed different pH optima for germination.

C. HEAT INDUCED DORMANCY

Brachfeld (1955) found that subjecting spores of Bacillus stearothermophilus 1518 to temperatures of 55° to 85°C would result in a significant reduction in plate counts. He called the phenomenon "heat-induced dormancy". Heat induced dormancy can be differentiated from heat injury, since these spores can be reactivated when re-exposed to activating temperatures. The effect was later confirmed by Finley and Fields (1962) in two strains of B. stearothermophilus. They found with spores of B. stearothermophilus strain 1518, greatest reduction in counts (65%) was achieved at 90°C whereas strain M showed greatest reduction in counts (10 - 15%) at 100°C.

Jaye and Ordal (1966) found spores of B. megaterium heated at 60°C in the presence of 40 mM calcium-dipicolinate became dormant. The control spores germinated completely in 40 mM calcium-dipicolinate at 24°C. Spores suspended in water, in a solution of sodium chloride, or sodium-dipicolinate and heated to 60°C would germinate when resuspended in calcium-dipicolinate at 24°C. The mechanism of calcium-dipicolinate induced dormancy at 60°C is not known, spores which failed to germinate in 40 mM calcium-dipicolinate after exposure to this chelate at 60°C, germinated in n-dodecylamine.

D. PRODUCTION OF SPORE SUSPENSION OF UNIFORM AND REPRODUCIBLE HEAT RESISTANCE

The foregoing discussion showed the various responses a given spore suspension could have when heated. Consequently, a statement that the "decimal reduction time" (D value) of a spore suspension is 80 minutes is misleading without reference to the shape of the inactivation curve. The discussion also highlighted the problem involved in preparing spore suspension of uniform and reproducible heat resistance. Ideally, spore suspension for used as biological indicator should give simple exponential kill depicted in Figure 3, Curve A. This would enable the initial population size to be adjusted such that complete kill was achieved at the end of the sterilization process. Moreover, the sterilization effect can be roughly assessed by monitoring the fall in colony counts using two selected points on the inactivation curve. The use of spore suspension exhibiting curve of Figure 3, Curve C, would result in the majority of the population being kill too soon in the course of

sterilization. A good biological indicator should ideally be destroyed just before the end of the sterilization process.

Spore suspension exhibiting biphasic or triphasic inactivation curve arise partly because of the continuous alteration of medium composition during batch cultivation. Spores formed early in the sporulation process may have preferentially consumed most of the medium components needed for the attainment of optimal heat resistance, leaving the residual nutrient for the rest of the sporulating members. The inherent non-reproducibility of batch cultivation data due to continual physiological change of the population from the moment of inoculation to the moment of harvesting has been pointed out by Tempest (1970). The advantages of using continuous cultivation (if possible) for spore production have been discussed in Section 2. D. Alternative method for preparing homogenous suspension of reproducible heat resistance by the endotrophic process coupled with continuous cultivation has also been discussed in Section 2. E.

Traditional thermobacteriology has assumed spore heat resistance to be a constitutive property, once "matured" in a culture, a spore is considered to have a definite fixed resistance in a given heating medium. Friesen and Anderson (1974) showed that this was not the case with B. stearothermophilus spores. They showed that spore suspension that did not exhibit straight line exponential kill at 110°C can be converted to straight line exponential kill by acid-stripping and converting them to calcium-spores, a method developed earlier on by Alderton and Snell (1963).

It seems therefore, that several approaches can be made to obtain spore suspension of uniform and reproducible resistance. The merit of each method used need to be studied further .

E X P E R I M E N T A L

1. MATERIALS

A. LIST OF ORGANISMS

B. stearothermophilus NCIB 8157, NCIB 8919, NCIB 8920 were obtained from The National Collection Of Industrial Bacteria, Torry Research Station, P. O. Box 31, Aberdeen, Scotland. These organisms cause flat-sour spoilage of canned foods. Spore suspensions of *B. stearothermophilus* NCIB 8919 are often used in testing steam sterilizers (Kelsey, 1961).

B. stearothermophilus NCTC 10,003 (wild type) was obtained from The National Collection Of Type Cultures, Central Public Health laboratory, Colindale Avenue, London NW9. The spores of this strain are used as biological control in steam sterilization.

B. stearothermophilus NCTC 10,003 (mutant) was obtained from Professor R. A. Anderson, Department of Pharmacy, University of Sydney, 2006, Australia. This strain was chosen for detailed studies because it grew in simple medium containing only glucose-mineral salts.

B. MEDIA

Glucose-tryptone agar: This medium contains 0.5% glucose, 1.0% tryptone and 2.0% agar. The medium was sterilized by autoclaving at 121°C for 15 minutes.

Chemically defined media: This was formulated after

careful studies of the organism nutritional requirements. Separate medium was used for the cultivation of B. stearothermophilus NCIB 8919, NCIB 8920, NCTC 10,003 (wild type) and NCTC 10,003 (mutant) as described in Section 3.

C. CHEMICALS

All the chemicals used were of AnalaR grade except $FeCl_2$ and amino acids. $FeCl_2$ was laboratory grade reagent. Amino acids were stated by the manufacturers to be chromatographically homogeneous. Lysozyme (egg white) with a potency of 25,000 units/mg. and trypsin with a potency of $\pm$ 0.5 Anson unit/g. at 35.5° (Anson, 1938) were obtained from British Drug Houses Ltd., Poole, Dorset. Dipicolinic acid (99.5%) was obtained from Koch-Light Laboratories., Colnbrook, Bucks. Polyethylene glycol 200 (PEG 200) was obtained from Grant Instruments (Cambridge) Ltd., Barrington, Cambridge. HEPES buffer: N-2-hydroxyethyl-piperazine-N'-2 ethanesulphonic acid (HEPES) at a concentration of 0.03 M. was used as medium buffer in experiments in which phosphate buffer was removed. This compound was obtained relatively pure ($>$ 99%) from Sigma London Chemical Company Ltd., Norbiton Station Yard, Kingston-upon Thames, Surrey KT2 7BH. A stock solution of buffer was normally prepared and the pH adjusted to 7.0 by adding AnalaR sodium hydroxide. HEPES stock was sterilized by filtration before incorporation into the final medium. All other chemicals were obtained from British Drug Houses, Hopkins and Williams

Ltd., Chadwell Heath, Essex or Fisons Scientific Apparatus Ltd., Bakewell Road, Loughborough, Leics.

Water was deionised prior to glass distillation.

D. APPARATUS

A Unicam S.P.600 spectrophotometer was used for optical density measurement. Atomic absorption spectroscopy was carried out using Unicam S.P.90. Spectrophotometers were obtained from Unicam Instruments Ltd., York Street, Cambridge. A pH meter, Pye model 290 was also obtained from Unicam Instruments Ltd. The International refrigerated centrifuge model B20 was supplied by The International Equipment Company, 300 Second Avenue, Needham Heights, Mass., 02194, U.S.A. The Davis Differential Cathode-ray Polarograph, Southern Analytical A1660 was bought from Southern Analytical Ltd., Frimley Road, Camberley, Surrey. The Mickle reciprocating shaker bath was obtained from Camlab Ltd., Nuffield Road, Cambridge, CB4 1TH. The Wild model B20 binocular phase contrast microscope was obtained from Micro Instruments (Oxford) Ltd., 7, Little Clarendon Street, Oxford, OX1 2PH. The high temperature thermostatic bath, model HB 10X was obtained from Grant Instruments (Cambridge) Ltd. The manufacturer claimed that the sensitivity of this instrument was $80^{\circ}C \pm 0.07$ and $180^{\circ}C \pm 0.06$ when used with PEG 200. All filtration equipment were obtained from Millipore U.K. Ltd., Heron House, Wembley, Middlesex. MLA automatic pipettes and tips were obtained from Frost Instrument Ltd., Workingham, Berkshire RG11, 1BG.

Haemocytometer counting chambers and Helber slides were obtained from Hawksley and Son Ltd., 12, Peter Road, Lancing, Sussex. All chambers complied with the British Standard Specification 748 (1963). 'BIOFLO' bench top chemostat, model C30 was obtained from New Brunswick Scientific Co. Inc., 1130, Somerset Street, New Brunswick, New Jersey 08903. The apparatus was recommended by the manufacturer to be suitable both for continuous and batch cultivation.

2. BASIC EXPERIMENTAL METHODS

A. TREATMENT OF GLASSWARE

Glassware was thoroughly soaked in 5% (v/v) solution of Decon 90 (obtainable through British Drug Houses Ltd.) overnight, rinsed once in distilled water and immersed in 1% (v/v) hydrochloric acid. This was followed by a further six rinses in distilled water and twice in deionised distilled water before drying. Glassware was sterilized by closure with aluminium foil, followed by heating at 160°C for 1 hour.

B. TREATMENT OF MEMBRANE FILTERS

All membrane filters used were previously boiled in three changes of deionised distilled water. This method effectively removed wetting agents and other chemicals which may contribute to the light absorption of liquids filtered through the membrane (Brown, Farwell and Rosenbluth, 1969).

C. PREPARATION OF SPORE STOCK

All the organisms were originally obtained as freeze-dried type cultures in ampoules. On receipt, they were cultured in glucose-tryptone broth overnight at 55°C for Bacillus stearothermophilus NCIB 8919, NCIB 8920 and NCTC 10,003 (wild type) and at 60°C for NCTC 10,003 (mutant). The over-night culture was streaked out onto glucose-tryptone agar from which an isolated colony was picked to initiate a second broth culture. Spore stock of each strain was prepared by inoculating

glucose-tryptone agar plates with cells from the second broth culture. Plates were incubated at 55°/60°C for 2-3 days when growth was washed off using glass spreaders. The method of Long and Williams (1958) was used to separate vegetative cells from spores (see below).

Spore stock was stored at 0-4°C in a refrigerator. Initially, spore stock was used to prepare inocula as described in Section 2.F. This was later abandoned and spores prepared from chemically defined medium was used instead. The procedure avoided the possibility of carry over of nutrients from complex into defined medium.

D. COLONY COUNTS

Viable counts were performed by spreading 0.5 to 1.0 ml. aliquots of diluted culture or spore suspension on the surface of five overdried glucose-tryptone agar plates. Serial dilutions were made in glucose-tryptone using an MLA pipette and were designed to give 30-200 colonies per plate after incubation. Incubation was done at 60°C for 16 hours, no increase in colony count was observed on further incubation even with heated spore suspension. The viable count was calculated from the mean colony count per plate.

The reproducibility of the counting procedure was tested by performing nine replicate counts on a stock suspension of spores. The results were tested by variance analysis as shown in Tables 2 and 3.

TABLE 2

ANALYSIS OF VARIANCE OF NINE REPLICATE COLONY COUNTS

COUNTS / PLATES	1	2	3	4	5	6	7	8	9	MEAN
A	284	246	221	226	227	253	198	219	246	235.5
B	276	158	254	232	201	255	286	296	228	242.8
C	268	184	200	221	222	236	273	258	240	233.5
D	157	269	188	287	249	215	287	289	243	242.6
E	139	228	243	232	227	215	191	276	280	225.6
T	1124	1085	1106	1198	1126	1174	1235	1338	1237	
$\bar{x}$	224.8	217.0	221.2	239.6	225.2	234.8	247.0	267.6	247.4	

(1) $\sum x^2 = 2985681$

(2) $\sum \frac{T^2}{n} = 2518166.2$

(3) $\frac{\sum x^2}{N} = 2507736.2$

n = Number of observations per count = 5

N = Total number of observations = 45

TABLE 3

ANALYSIS OF VARIANCE OF NINE REPLICATE COLONY COUNTS

SOURCE OF VARIANCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES
BETWEEN COUNTS	2) - 3) = 10430	m - 1 = 8	1303.75
WITHIN COUNTS	1) - 2) = 467514.8	mn - m = 36	12986.5

m = number of counts = 9

F = variance ratio = 1303.75/12986.50

= 0.10

Tabulated value of F for 8/36 degrees of freedom at 1% significance is 3.08. Therefore, the variation between counts is not significantly greater than the variation within counts.

The coefficient of variation of the means was 3.0% (within counts) and 6.8% (between counts).

E. TOTAL COUNTS

Total counts were performed using haemocytometer counting chambers, having a chamber depth of 0.1 mm. and Improved Neubauer rulings. Cook and Brown (1964) showed that chamber of this depth gave less variation in counts than chamber of 0.02 mm. depth.

Spore suspensions were agitated with glass balls and then diluted where necessary with membrane-filtered water such that each small square on the grid contained approximately 5-10 spores. The suspension was introduced into the chamber by running it beneath the cover slip with a Pasteur pipette, the cover slip having been first pressed down until "Newton's Rings" could clearly be seen on areas of contact.

If air bubbles were introduced the slide was discarded. The filled slides were stored in a petri dish containing moist cotton wool for about 30 minutes before counting. This allowed the spores to sediment onto the platform.

The spores in 60 pre-selected small squares were counted using a phase contrast microscope at a magnification of 600x. About 300 spores per slide were counted each time.

The reproducibility of the counting procedure was tested using four slides to make sixteen replicate counts on a spore suspension. The results are shown in Tables 4 and 5.

TABLE 4

ANALYSIS OF VARIANCE OF SIXTEEN REPLICATE TOTAL COUNTS

(The figures denote mean number of spores per square).

SLIDES^m / REPLICATES^n	1	2	3	4	MEANS
A	8.038	7.738	8.700	8.275	8.188
B	7.975	8.650	8.050	8.900	8.394
C	7.888	8.813	8.563	7.362	8.157
D	8.300	8.575	8.313	8.538	8.432
T	32.201	33.776	33.626	33.075	
MEANS	8.050	8.444	8.407	8.269	

(1) $\sum x^2 = 1102.9300$

(2) $\frac{\sum T^2}{n} = 1100.5950$

(3) $\frac{\sum {}^2x}{N} = 1100.2157$

n = Number replicates per slide = 4

N = Total number of observations = 16

TABLE 5

ANALYSIS OF VARIANCE OF SIXTEEN REPLICATE TOTAL COUNTS

SOURCE OF VARIANCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES
BETWEEN SLIDES	2) - 3) = 0.37927	m - 1 = 3	0.1264
WITHIN SLIDES	1) - 2) = 2.3350	mn - m = 12	0.1946

F = VARIANCE RATIO = 0.1264/0.1946 = 0.6495

Tabulated value for F at 3/12 degrees of freedom at 1% significance is 5.95. Therefore, the variation between slides is not significantly greater than the variation within slides at 1% probability level.

From the results in Table 4, the coefficient of variation of the means was 1.7% (within slides) and 2.1% (between slides).

F. BATCH CULTIVATION OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

B. stearothermophilus NCTC 10,003 (mutant) was batch cultivated at 60°C using 'BIOFLO' bench top chemostat, model C30. This apparatus was recommended by the manufacturer to be suitable for use both for continuous and batch cultivation.

The chemical composition of the medium used was described in Section 5. The medium was modified appropriately in accordance with the object of the experiment as described in Section 6. 350 ml. of medium contained in a culture vessel was aerated with sterile air (1.2 litre/litre medium/minute) fed through a filler packed with fiber glass. To reduce the loss of water from culture during air-sparging, the air bubbled into the medium was water-saturated first by introduced into an air-washing flask, containing sterile distilled water. Rapid mixing and agitation of the medium was done by an impeller running at a speed of 400 r.p.m. Experiments had shown this level of aeration to be optimum for sporulation (Section 5).

The inoculum consisted of an overnight culture grown in chemically defined medium containing a limiting concentration of the nutrient component under investigation and passaged twice through a medium of similar composition.

With the exception of phosphate depleted growth, cultures were grown in media containing a sufficient concentration of

the depleting nutrient to support growth to an optical density of 1.0 at 420 nm. This ensured that the oxygen availability per cell was similar with the different nutrient depletion experiments. For phosphate depleted growth, the culture was grown to an optical density of 0.75 since the maximum optical density under the experimental conditions was 0.8 (see Figure 37).

C. METHOD FOR HARVESTING AND CLEANING OF SPORES

Cultures were grown overnight and harvested by centrifugation, 4-5 hours after maximum growth and the completion of sporulation. The cells were washed twice with sterile, deionised distilled water by repeating centrifugation and resuspension. Finally the cells were resuspended in 150 ml. of deionised distilled water.

Spores were cleaned by the procedure of Long and Williams (1958). In this method, lysozyme was added to the cell suspension to give a final concentration of 200 I.U./ml. and the suspension was incubated at 37°C for 2-3 hours, until all the vegetative cells were lyzed. The suspension was centrifuged at 800 x g for 15 minutes in a refrigerated centrifuge. The supernatant was poured off. The upper layer of the pellet, consisting mainly of phase-dark spores and undigested cell debris was washed off and discarded; the remaining portion of the pellet was again suspended in water and centrifuged as before. In this way, phase bright spores were separated

from the vegetative cell materials. The spores were finally washed and resuspended in 0.05 M. saline. It was found that washing and storing of spores in dilute saline overcame spore clumping. The spore suspension was stored at 0-4°C until use.

H. DETERMINATION OF PERCENTAGE SPORULATION

The percentage sporulation of a culture was determined by directly counting the number of refractile spores and sporangia produced after a suitable period of incubation.

The Helber bacteria counting chamber (Hawksley and Son Ltd.) having a Thoma ruling and a depth of 0.02 mm. was used with a B.S.748 cover glass. This type of chamber was used in preference to haemocytometer counting chambers of depth 0.1 mm. because of their reduced thickness and chamber depth, thus giving better focussing and phase contrast.

Samples were first diluted with a filtered solution of dilute formaldehyde to give approximately 8-12 organisms per small square of the grid. The concentration of formaldehyde in the final diluted culture was 1.0% (w/w). The culture was then run under cover glass using a Pasteur pipette and the percentage of phase-bright spores scored using a phase-contrast microscope. A x 40 objective used in conjunction with a x 15 eyepiece (giving 600 x magnification) was generally suitable for the evaluation of percentage sporulation of B. stearothermophilus culture.

TABLE 6

REPRODUCIBILITY OF PERCENTAGE SPORULATION DETERMINATION

COUNT NUMBER	% SPORULATION
1	39.7
2	42.4
3	39.8
4	40.2
5	43.4

$$\text{Standard deviation} = \sqrt{\frac{\Sigma x^2 - (\Sigma x)^2/n}{n - 1}}$$

$$= 0.42$$

$$\text{Coefficient of variation} = \frac{s}{\bar{X}} \times 100$$

$$= 1.0\ \%$$

The reproducibility of the method was assessed by performing five replicate counts on the same culture and the results analysed statistically as shown in Table 6. It can be seen that good reproducibility was achieved.

I. ASSAY OF HEXOSAMINE

Hexosamine was assayed using the method of Good and Bessman (1964). This method used borate buffer for the modification of Elson-Morgan and Morgan-Elson reactions. Preliminary studies revealed that the chromogen formed in Elson - Morgan and Morgan-Elson reactions, absorbed maximally at wavelength of 580-585 nm. instead of 570 nm. recommended by the authors (Figure 4).

A series of standard solutions of glucosamine-HCl with concentrations ranging from 10-82 µg./ml. was made in distilled water. A calibration curve was constructed according to the method described by Good and Bessman (1964) Figure 5 shows a representative calibration curve. A linear relationship between optical density and concentration of glucosamine was obeyed over the range of concentrations used. A calibration curve was constructed on each occasion the assay was performed. Tests and standards were run simultaneously and the concentrations of hexosamine in tests determined directly from the calibration curve

FIGURE 4

THE ABSORPTION OF CHROMOGEN FORMED IN MODIFIED EISON-MORGAN REACTION

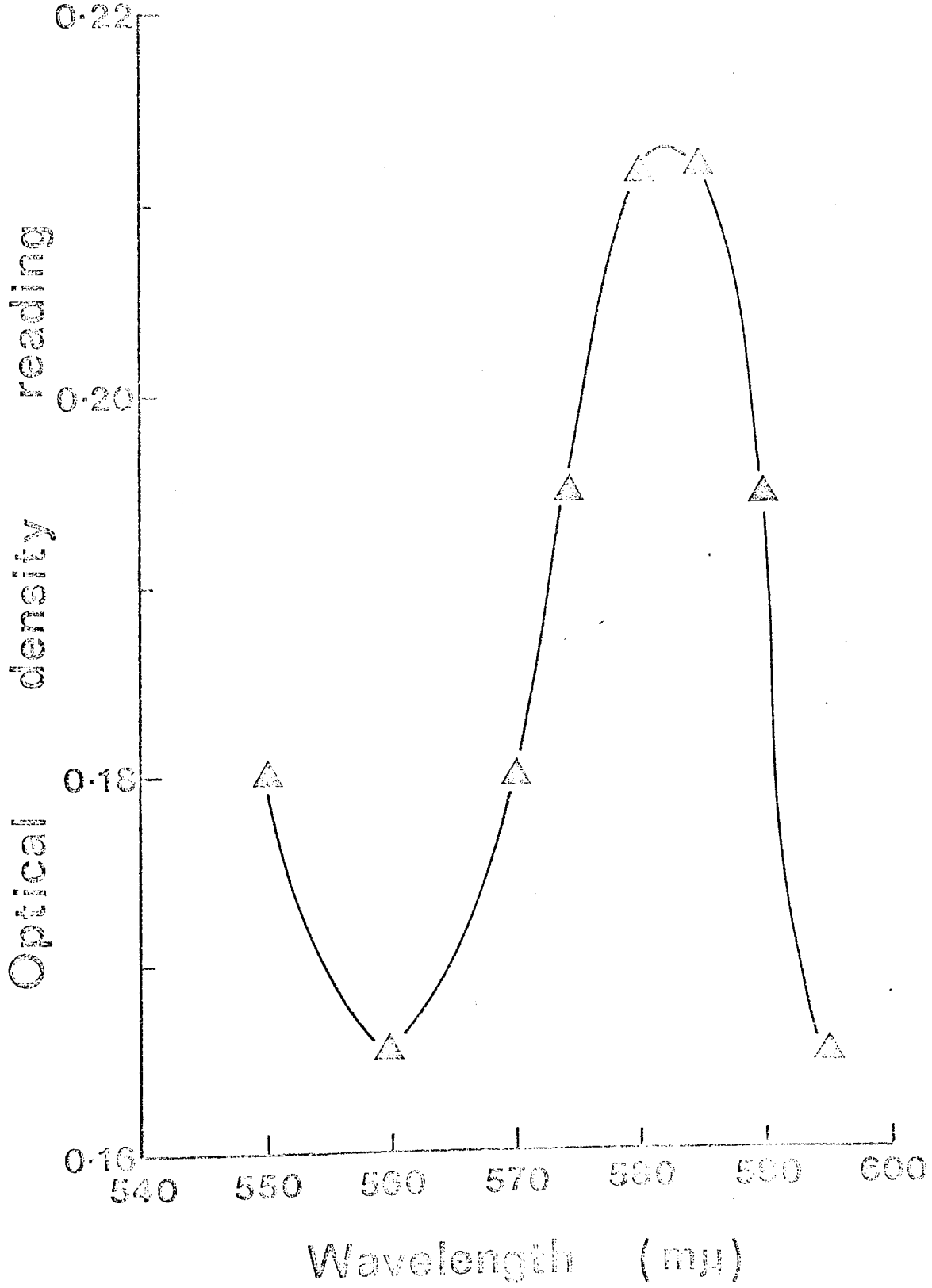
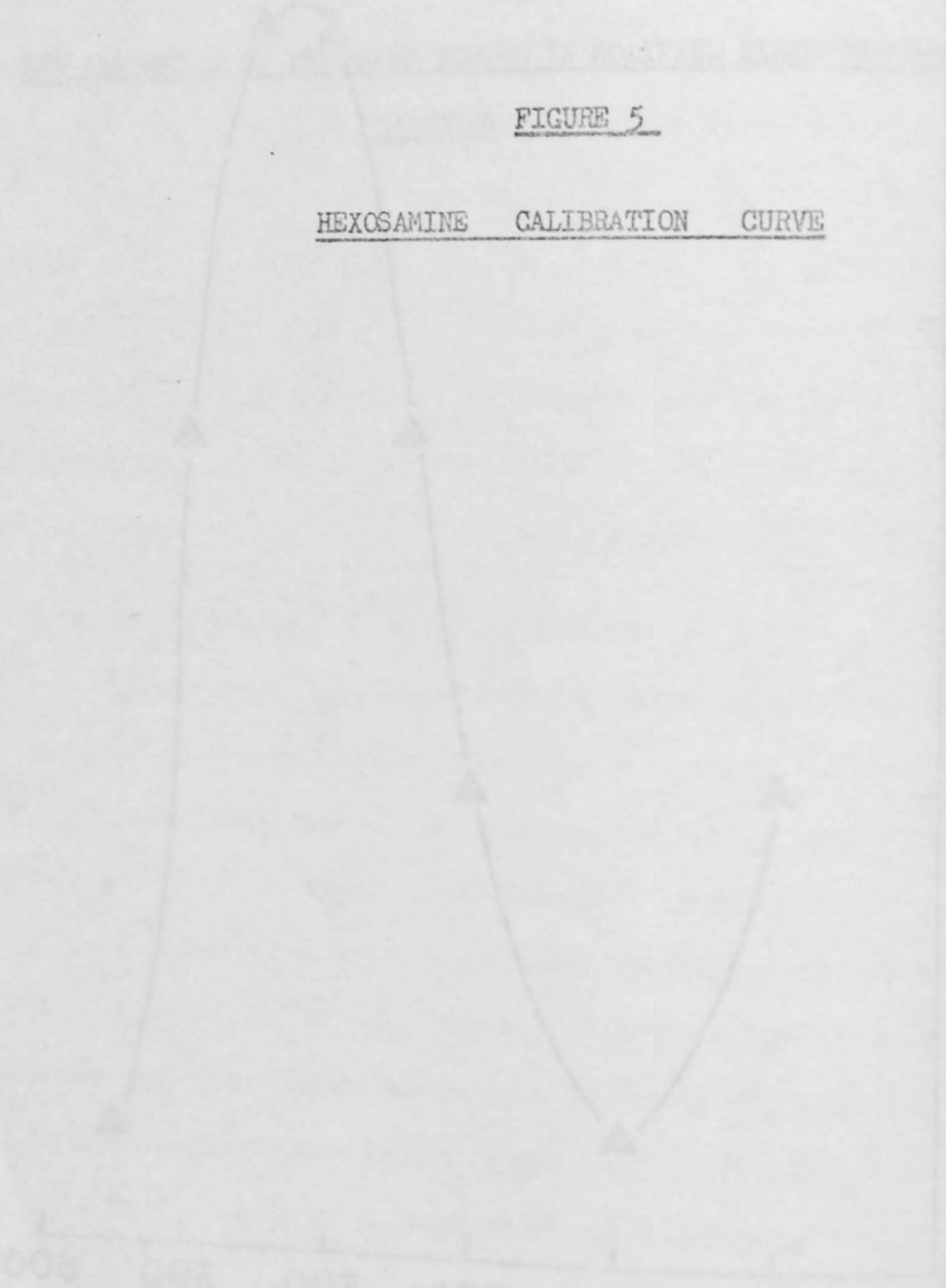
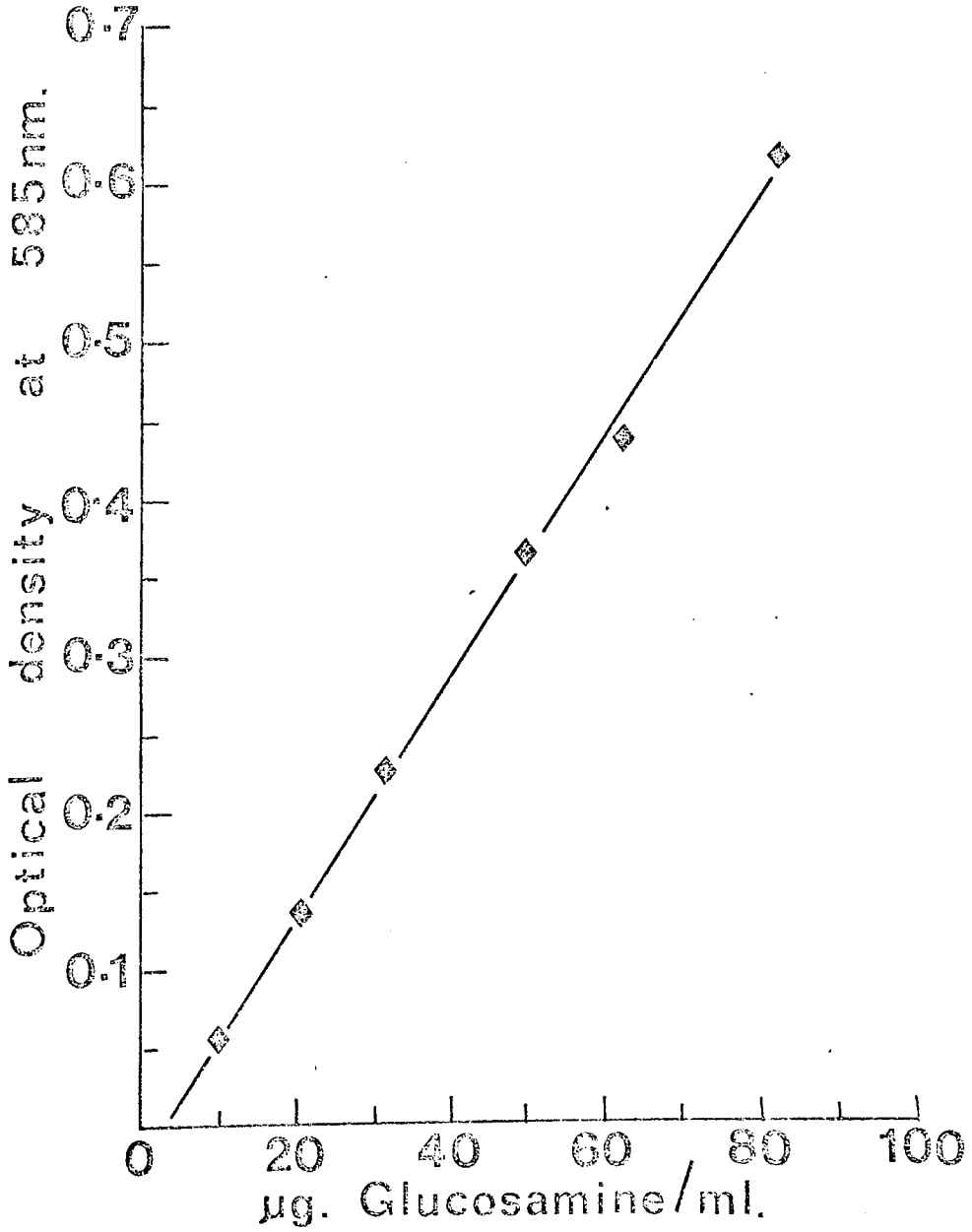

Optical density reading
0·22
0·20
0·18
0·16
540
550
560
570
580
590
600
Wavelength (mμ)

FIGURE 5

HEXOSAMINE CALIBRATION CURVE




Optical density at 585nm.
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
20
40
60
80
100
µg. Glucosamine/ml.

(a) Reproducibility of Glucosamine Assay

The reproducibility of the assay was assessed by measuring the concentrations of hexosamine in replicate test samples. The test sample was prepared by hydrolysing 1.0×10^8 spores (originally in 0.05 N. saline) in 10 ml. of 6 N. HCl at 100°C for 5 hours (Murrell and Warth, 1965). Two controls each containing the equivalent amount of 0.05 N. saline as the tests were similarly prepared. On the completion of acid hydrolysis the acid was removed by evaporation in a rotatory evaporator. The residue was washed three times with distilled water by repeating dissolution and evaporation. The residue was finally dissolved in 1 ml. of water and assayed for hexosamine as described by Good and Bessman (1964). The concentration of hexosamine in tests tended to be low, giving rise to low optical density readings and hence the inaccuracy of the measurements. This problem was overcome by the use of internal standard. In the present study, an internal standard of 31.1 µg. glucosamine was used in all test samples.

The reproducibility of the method is shown in Table 7.

J. ASSAY OF DIAMINOPIMELIC ACID (D.A.P.)

Diaminopimelic acid (D.A.P.) was assayed by the method of Gilvarg (1958). The author used a sample volume of 0.13 ml. This volume could not be accurately measured in the present study. Consequently, sample volume of 0.2 ml. was used and the volume of conc. HCl/ninhydrin/propanol-water mixture were

TABLE 7

THE REPRODUCIBILITY OF HEXOSAMINE ASSAYS

TEST NUMBER	µg. HEXOSAMINE / ml.
1	58
2	60
3	59
4	55
5	68

$$\text{Standard deviation} = \sqrt{\frac{\Sigma x^2 - (\Sigma x)^2/n}{n - 1}}$$

$$= 4.8$$

Coefficient of variation = 8.0%.

correspondingly adjusted to 0.11 ml./0.62ml./6.76ml.

A series of standard solutions of D.A.P. with concentrations ranging from 5 to 110 µg./ml. was prepared in distilled water. A calibration curve was constructed according to the method described by Gilvarg (1958). Figure 6 shows that linear relationship between optical density and concentration of D.A.P. was obeyed over the range of D.A.P. used. The reason for the intercept on the Y-axis is not known.

A calibration curve was constructed on each occasion the assay was performed. Tests and samples were run simultaneously and the concentrations of D.A.P. in tests determined directly from the calibration curve.

The reproducibility of the assay was assessed by measuring the concentration of D.A.P. in replicate test samples, prepared by hydrolysing 1.0×10^8 spores in 10 ml. of 6 N. HCl at 110°C for 20 hours (Murrell and Warth, 1965). All other details were as described for glucosamine assay. Internal standard of 13.0 µg. D.A.P. was added to all test samples. The reproducibility of the method is shown in Table 8 after accounting for the readings in the controls.

FIGURE 6

DIAMINOPIMELIC ACID CALIBRATION CURVE

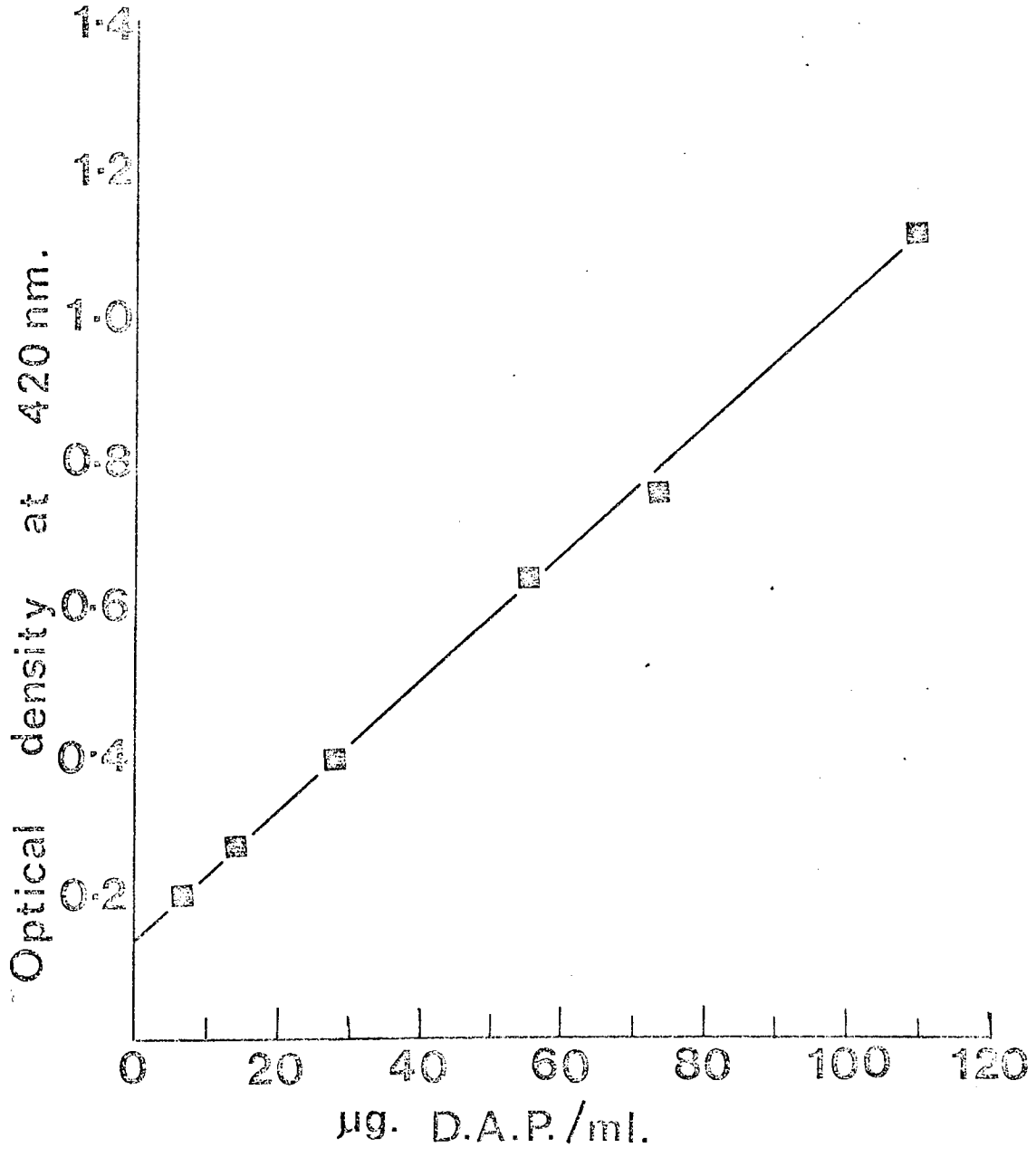

Optical density at 420 nm.
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
20
40
60
80
100
120
µg. D.A.P./ml.

TABLE 8

THE REPRODUCIBILITY OF DIAMINOPIMELIC ACID ASSAY

TEST NUMBER	µg. D.A.P./ml.
1	7.0
2	6.5
3	6.5
4	7.0
5	7.0

Mean = 6.8

Standard deviation = 0.27

Coefficient of variation = 4.0%

K. ASSAY OF DIPICOLINIC ACID (D.P.A.)

Dipicolinic acid was assayed using a Davis differential cathode-ray polarograph (Southern Analytical A1660) according to the method of Porter et al (1967). A series of standard solutions of D.P.A. with concentrations ranging from 5 to 40 µg./ml. was made with 0.5 N. HCl or citrate buffer pH 4 and direct polarographic measurements were made to obtain a calibration curve (Figure 7). The instrument was calibrated on each occasion of use. The standards and unknowns were each read three times and the average values determined.

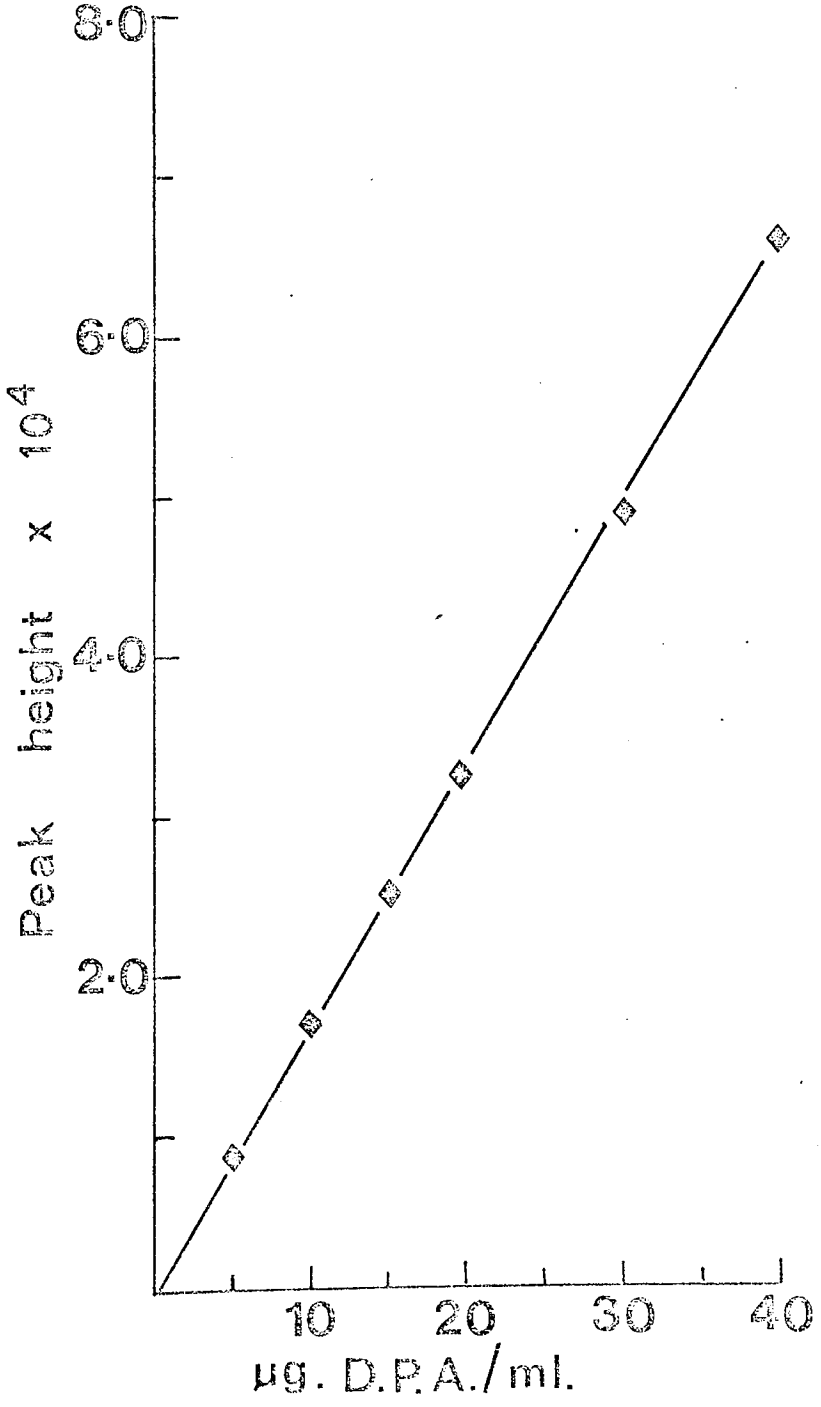
The calibration curve made with D.P.A. in 0.05 N. HCl was used to read the concentration of unknown in a similar suspending medium, for example, in the determination of total D.P.A. in spores when they were acid-hydrolyzed with 0.5 N. HCl. Likewise, calibration curves made with D.P.A. in citrate buffer, pH 4 were used to read the concentration of unknowns in filtrates in experiment involving the release of D.P.A. during heating in citrate buffer at pH 4 (see Section 2.P.). In this way, variation in readings due to different suspending media was avoided.

L. ASSAY OF CALCIUM, MAGNESIUM AND MANGANESE

The calcium, magnesium and manganese contents of samples were determined using atomic absorption spectroscopy. Willis (1960) recommended the inclusion of 10,000 µg./ml. lanthanum ions in order to overcome the suppression of calcium

FIGURE 7

DIPICOLINIC ACID CALIBRATION CURVE


8·0
6·0
4·0
2·0
Peak height x 10⁴
10
20
30
40
µg. D.P.A./ml.

absorption caused by phosphate ions. The effectiveness of La^{3+} in overcoming the suppression of Ca^{2+} absorption by phosphate ions was studied by Hodges (1973). He found La^{3+} (10,000 µg./ml.) was effective in preventing suppression of Ca^{2+} absorption for phosphorus concentrations up to (at least) 25 µg./ml. ($\equiv$ c. 75 µg./ml. PO_4^{3-}). In the present study, lanthanum ions were added to all samples measured, to a final concentration of 10,000 µg./ml.

The instrument (Unicam S.P.90) was set to the conditions described by the manufacturer during measurement. For each set of determination, test solutions were compared with standard calibration solutions run at the same time. Each sample was read three times and the average value determined. Standard solutions were adjusted to give reading lying between 90% and 30% transmission (0.046 to 0.523 optical density) which is the accurate range of the instrument. Calibration curves for calcium, magnesium and manganese are shown in Figures 8 and 9. All three lines exhibited some curvatures.

M. THE REPRODUCIBILITY OF DIPICOLINIC ACID; CALCIUM; MAGNESIUM AND MANGANESE ASSAYS

Stock suspension of spores (in 0.05 N. saline) was vigorously agitated with glass balls to disperse any clumps that might form during storage. The suspension was diluted with hydrochloric acid to give a final concentration of $\sim 7 \times 10^7$ spores/ml. in 0.5 N. HCl. 5 ml. of this suspension was sealed in each of

FIGURE 8

MAGNESIUM AND CALCIUM CALIBRATION CURVE

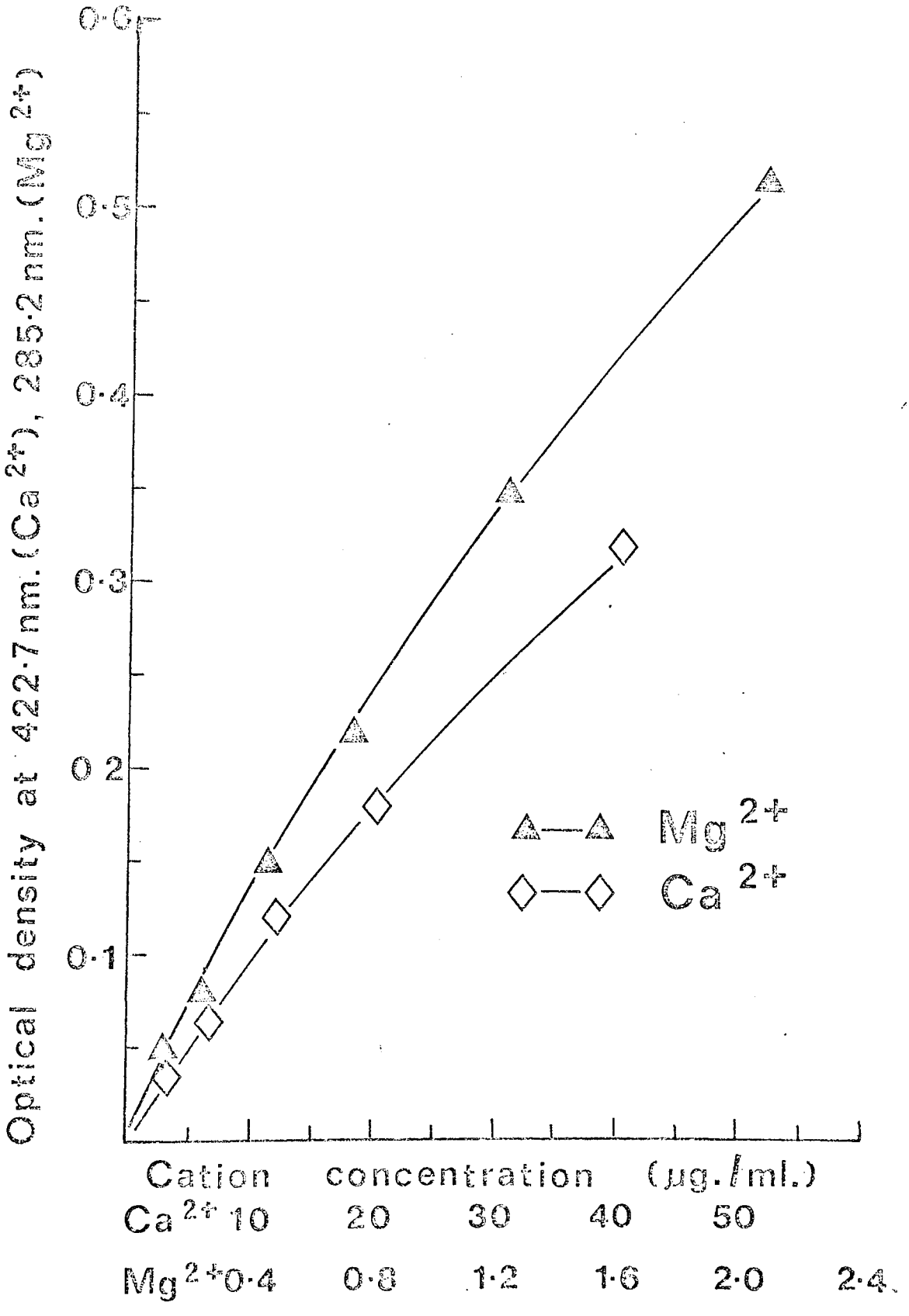
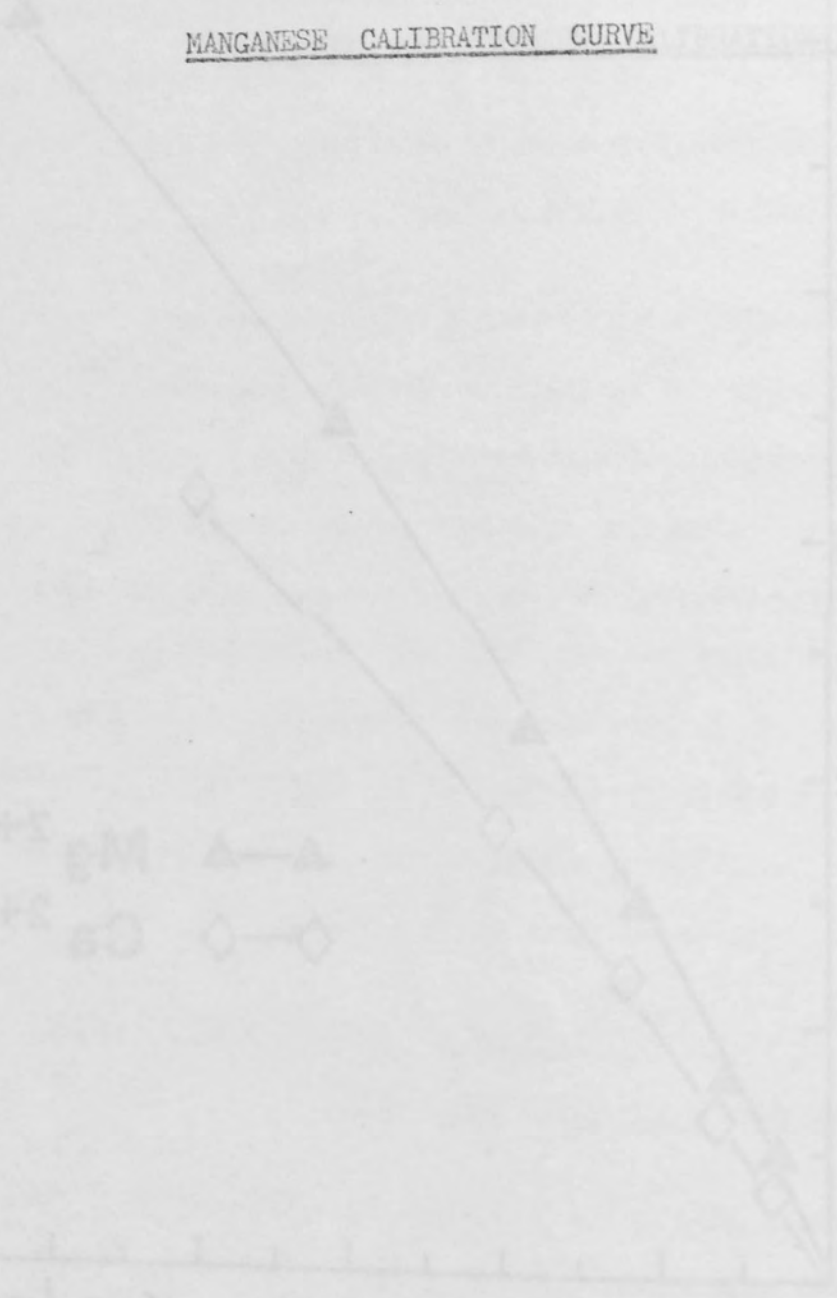

Optical density at 422·7 nm. (Ca^{2+}), 285·2 nm. (Mg^{2+})
0·6
0·5
0·4
0·3
0·2
0·1
Mg^{2+}
Ca^{2+}
Cation concentration (µg./ml.)
Ca^{2+} 10 20 30 40 50
Mg^{2+} 0·4 0·8 1·2 1·6 2·0 2·4

FIGURE 9

MANGANESE CALIBRATION CURVE



Optical density at 279·5 nm.
0·4
0·3
0·2
0·1
0
1
2
3
4
5
µg. Mn2+3/ml.

3 x 5 ml. glass ampoules previously cleaned with dilute hydrochloric acid. Two control ampoules each containing 5 ml. of 0.05 N. saline in 0.5 N. HCl were also prepared. The ampoules were autoclaved at 121°C for 1 hour and the contents assayed for D.P.A. as described in Section 2.K.

Since polarographic measurement of D.P.A. does not destroy the sample, the sample was used for cation assays. 4 ml. of the solution was withdrawn from the polarographic cell and to it was added 1 ml. of 5% (w/v) lanthanum solution. The Ca^{2+}, Mg^{2+} and Mn^{2+} concentration of each of the tests and controls was determined. The Mn^{2+} content of spores tended to be low compared with Ca^{2+} or Mg^{2+}. Manganese determination was often carried out using scale expansion in a manner suggested by the instrument manufacturer.

The reproducibility of the various assays are presented in Table 9.

N. MEASUREMENT OF HEAT INACTIVATION

0.25 ml. of spore suspension was introduced into each of several sterile 1 ml. glass ampoules with an MLA automatic pipette connected to a sterile hypodermic needle. They were then sealed. Each ampoule was fastened to a wire and attached to weights. The ampoule was then totally immersed in a thermostatically controlled oil bath (Model HB 10X, Grant Instrument Ltd., Barrington, Cambridge) at one minute

TABLE 9

THE REPRODUCIBILITY OF DIPICOLINIC ACID, CALCIUM, MAGNESIUM AND MANGANESE ASSAYS

SAMPLE NUMBER	DPA. Conc. µg./ ml.	Ca^{2+} Conc. µg./ ml.	Mg^{2+} Conc. µg./ ml.	Mn^{2+} Conc. µg./ ml.
1	9.00	2.20	4.80	0.60
2	9.00	2.30	4.30	0.53
3	9.25	2.50	4.80	0.58
4	9.25	2.50	4.70	0.58
5	9.13	2.30	5.00	0.58
MEAN:	9.13	2.36	4.72	0.57
S.D.*	0.12	0.13	0.23	0.026
C.V.**	1.3%	5.5%	4.9%	4.6%

S.D.* = Standard deviation.

C.V.** = Coefficient of variation.

intervals to prevent possible drop in bath temperature. The "come-up times" was assumed to be short. Since this source of error was constant throughout the series of experiments, it should not affect the conclusion drawn from the results. Using a thermocouple, Cook and Brown (1964) found that 100°C was reached in less than 30 seconds, 115°C and 121°C in less than 50 seconds.

At selected times, measured by a stop watch, an ampoule was removed and immediately cooled by immersing in ice-cold water. Heated spore suspension was serially diluted with glucose-tryptone broth. 0.5 ml to 1.0 ml of suspension was spread onto the surface of five glucose-tryptone agar plates such that each plate gave rise to 30 - 300 colonies after incubation. Plates were incubated at 60°C and the number of colonies counted as described in Section 2.D. Death of a spore is defined as the loss of power to form a colony on glucose-tryptone agar. This medium was previously reported to give optimal recovery to severely heated spores of Bacillus stearothermophilus (Campbell et al., 1965).

The reproducibility of heat inactivation measurements was assessed by making duplicate determinations on a suspension of B. stearothermophilus spores (Figure 10). The linear portion of the inactivation curve was subjected to statistical analysis. The results showed no significant difference between the data of duplicate experiments.

FIGURE 10

REPRODUCIBILITY OF HEAT INACTIVATION CURVE OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM CARBON DEPLETED CULTURES

▽—▽ Experiment 1 $*y = (1.28 \times 10^6) - 0.0104x$

$r = -0.9993$

$P = < 0.001$ D.F. = 7

○—○ Experiment 2 $*y = (1.09 \times 10^6) - 0.00971x$

$r = -0.9973$

$P = < 0.001$ D.F. = 7

* Linear part of the curve

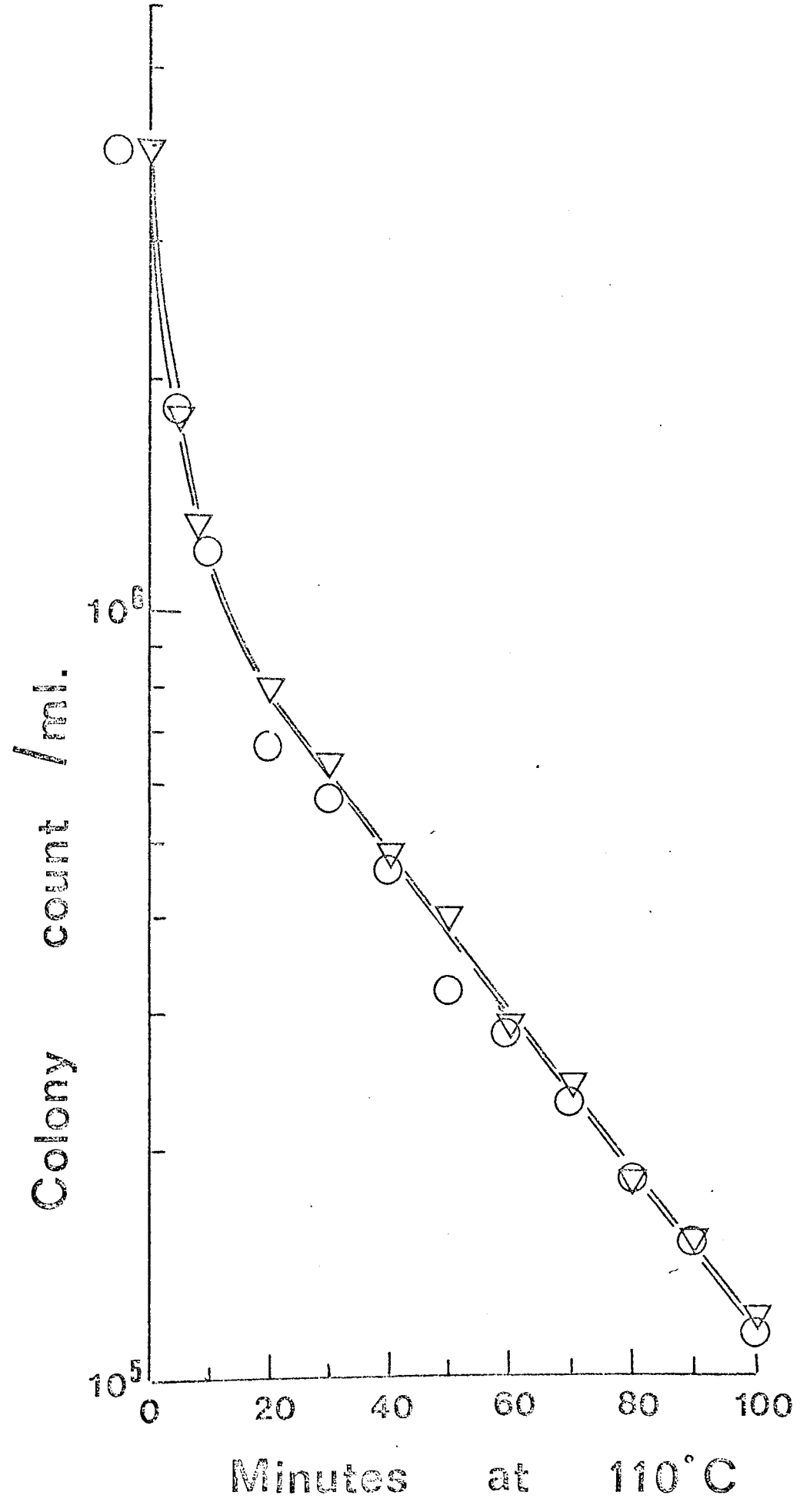

Colony count / ml.
10^6
10^5
0
20
40
60
80
100
Minutes at 110°C

FIGURE 11

REPRODUCIBILITY OF HEAT INACTIVATION CURVES OF THREE BATCHES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED FROM NITROGEN DEPLETED CULTURES

○—○ Batch 1 $y = (2.80 \times 10^6) - 0.00295x$

$r = -0.990$

$P = 0.001$ D.F. = 6

▽—▽ Batch 2 $y = (2.36 \times 10^6) - 0.00297x$

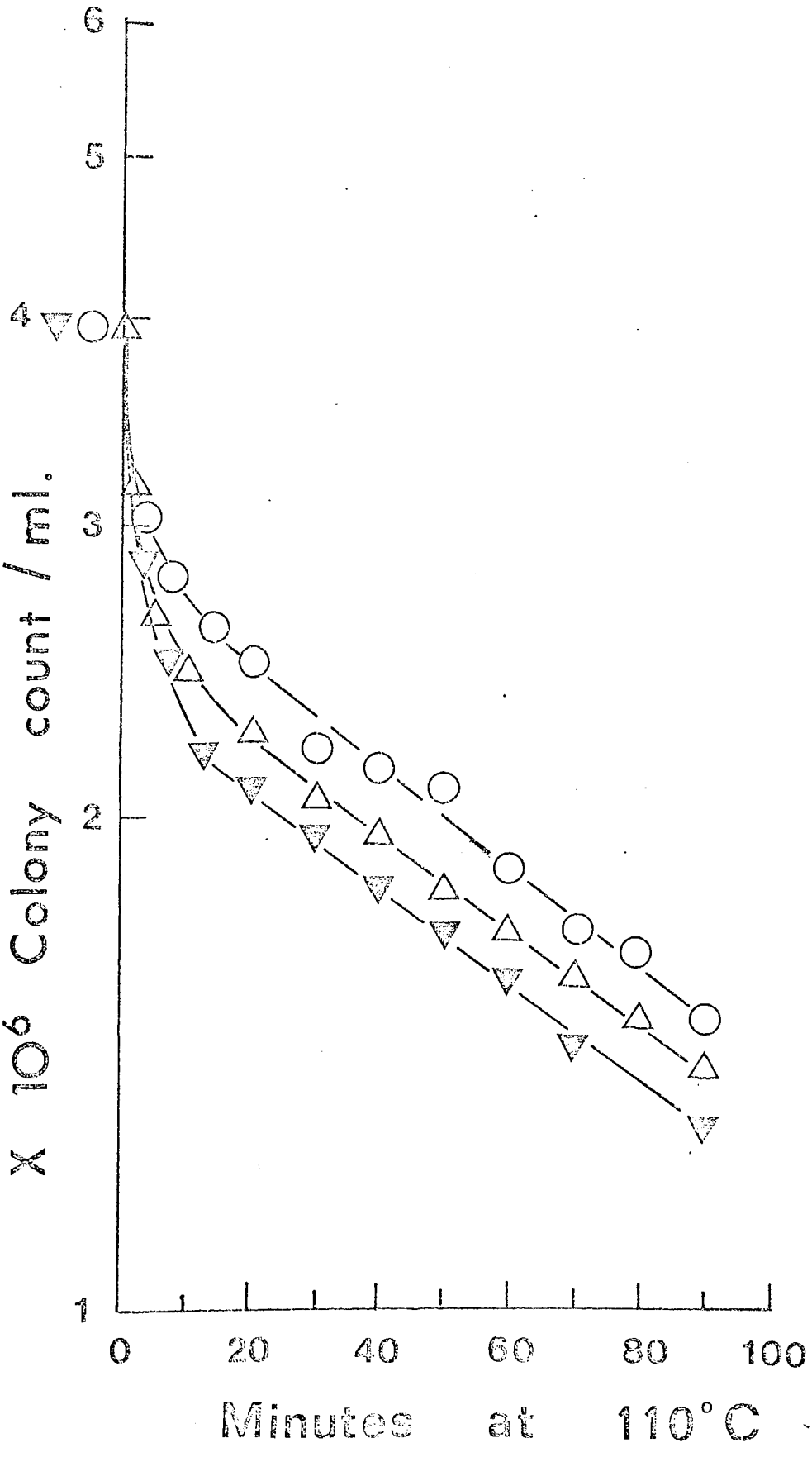
$r = -1.004$

$P = 0.001$ D.F. = 5

△—△ Batch 3 $y = (2.52 \times 10^6) - 0.00284x$

$r = -1.003$

$P = 0.001$ D.F. = 6


6
5
4
3
2
1
X 10⁶ Colony count / ml.
0
20
40
60
80
100
Minutes at 110°C

The variation between batches of spores of similar kinds is shown in Figure 11. It can be seen that the linear portion of the inactivation curve is reproducible. However, the proportion of heat sensitive to heat resistant population does vary from batch to batch. This variation may be attributed to the method of spore preparation which consisted of repeating removals of upper layer of pellet on centrifugation. It is possible that different proportions of heat sensitive to heat resistant population were removed from batch to batch.

O. MEASUREMENT OF THE EXTENT OF GERMINATION

The extent of spore germination was determined by measuring the rate of phase-darkening following incubation at 60°C. This method was used because spore suspension of B. stearothermophilus NCTC 10,003 (mutant) germinated slowly in germination solution (even of complex glucose-tryptone broth) when shaken in a reciprocating shaker. Attempt to increase the rate of germination by using heat activated spores was unsuccessful. This slow rate of germination may be attributed to the critical importance of aeration level in germination medium on the germination of B. stearothermophilus spores.

In the phase-darkening method, 15 ml. of glucose-tryptone agar was prepared in a 100 ml. beaker and sterilized by autoclaving at 121°C for 15 minutes. When

solidified, the agar was overdried at 60°C for 1 hour. 0.1 ml. of spores (about 1.0×10^8 spores/ml.) was then evenly spread on the surface of agar. When the liquid had been completely absorbed, the beaker was incubated at 60°C in a thermostatically controlled water bath. At various time intervals, microscope slides were prepared from pre-selected part of agar surface and the percentage of spores turned phase-dark scored using phase contrast microscopy.

Throughout the study, beakers of the same make were used to lessen the variation of beaker thickness which could affect the rate of heat penetration during incubation. This also ensured that the agar used was of uniform thickness between experiments. The reproducibility of the method was assessed by making replicate experiments. The results are shown in Figure 12 which shows a good degree of reproducibility was achieved.

P. MEASUREMENT OF DIPICOLINIC ACID AND CATION RELEASE FROM SPORES

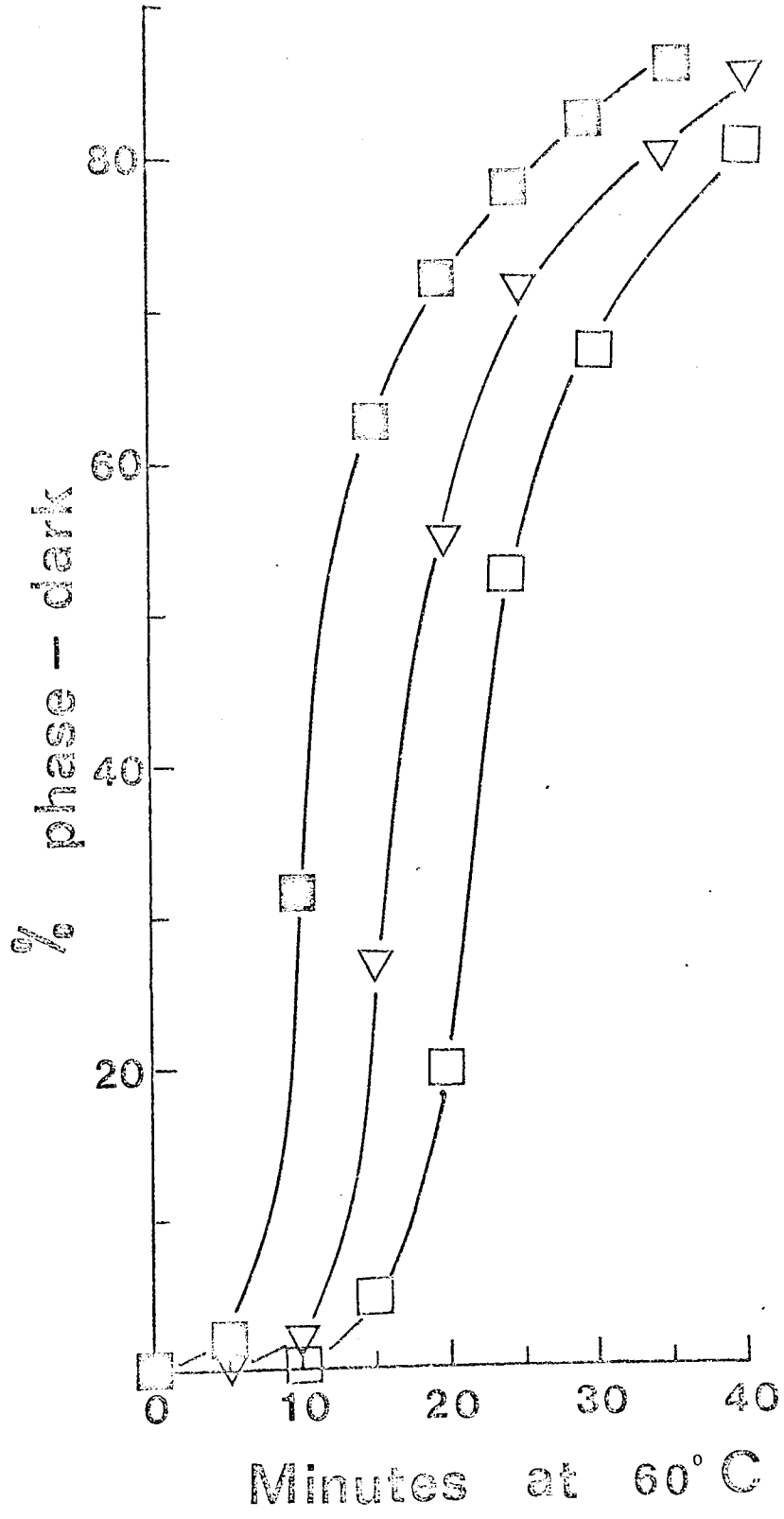
2.5 ml. of stock spore suspension ($6\text{-}10 \times 10^8$ spores/ml.) was diluted with 2.5 ml. of double strength Sorensens citrate buffer, pH 4 contained in each of the several 5 ml. glass ampoules, which had been previously boiled with dilute hydrochloric acid and rinsed several times with deionised distilled water. They were then sealed and the content

FIGURE 12

REPRODUCIBILITY OF GERMINATION PATTERN OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM NITROGEN DEPLETED CULTURES

	% Germination after 30 minutes
■—■ Experiment 1	83
▽—▽ Experiment 2	81
□—□ Experiment 3	81

Note: Time scale displaced by 5 minutes for each curve.


% blue - dark
80
60
40
20
0
10
20
30
40
Minutes at 60° C

mixed with a vortex mixer. Each ampoule was then fastened to a wire and attached to weights. The ampoules were totally immersed in a thermostatically controlled oil bath in a manner described for the measurement of heat inactivation (Section 2.N.)

At selected times, an ampoule was removed and immediately cooled by immersing in cold water. The ampoule was opened and the content filtered through a 0.45 μ pore size membrane contained in a Millipore Swinnex 25 disposable filter holder. The filtrate was subsequently assayed for D.P.A. and cations as previously described in Section 2.K. and 2.L.

Q. MEASUREMENT OF TURBIDITY

(a) Introduction

In general and with exceptions, the number of bacteria during growth can be determined with sufficient accuracy and great ease by estimating the degree of turbidity of culture.

When light passes through a bacterial suspension, part is scattered due to the reflection at the surface of the organisms and to diffraction within them. The total amount of light scattered increases directly with the ratio, particle size: wavelength of incident light. It follows that scattering will be greater (a) at a given wavelength, the larger the organisms, and (b) the shorter the wavelength used with a given organism (Meynell and Meynell, 1970). The total amount of light scattered is also dependent upon the

concentration of cells in the suspension, governing at low bacterial concentrations by the Lambert-Beer law:

$$\log_{10} \frac{I_o}{I} = s.c.l.$$

where I is the intensity of a parallel monochromatic beam of light after passing through the suspension; I_o is the incident intensity; $\log_{10} \frac{I_o}{I}$ is the optical density or extinction of the suspension; s is the turbidity coefficient depending on the particular organism; l is the length of the light path through the suspension and c is the bacterial cell concentration.

There is an inverse relationship between the turbidity or optical density of a bacterial suspension and the amount of light that passes through it. The light transmitted is measured accurately by the photoelectric cell of the instrument. In the present study, a Unicam S.P. 600 spectrophotometer was used for turbidimetric measurement. This instrument measures optical density over the wavelength range of 350-1000 nm. Matched 10 mm. quartz-cell cuvettes were employed to eliminate or standardize all variables except the number of bacteria.

(b) Choice of Wavelength

The use of a suitable wavelength is critical to all turbidimetric measurements. Preliminary studies showed that the S.P. 600 spectrophotometer was more sensitive to

changes in optical density as the wavelength was decreased. However, prolonged usage at low wavelength was discouraged by the deterioration of the light source and photocell, giving rise to loss of sensitivity eventually. Moreover, it was found that when an overnight culture was filtered and the culture filtrate read against fresh medium over the wavelength range of 360-500 nm., the background contribution to optical density due to the medium constituents and products of metabolism became negligible at wavelength greater than 400 nm. Also, the optical density of fresh medium read against distilled water was negligible at this wavelength. A wavelength of 420 nm. was finally selected.

(c) The Effect Of Cell Concentration On Optical Density

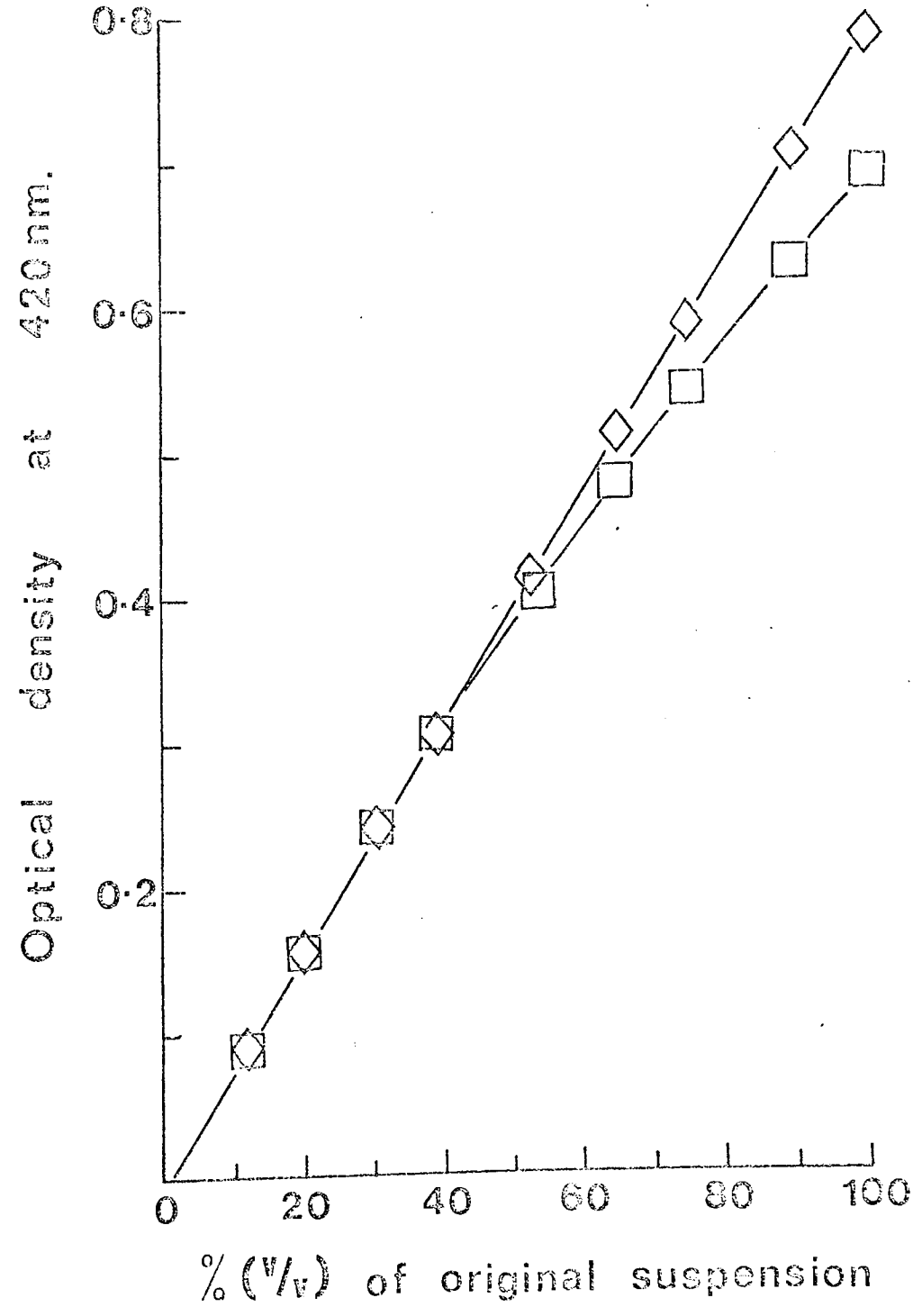
The effect of increasing cell concentration on optical density reading was studied by diluting a stationary phase culture of B. stearothermophilus in defined medium. A linear relationship between optical densities and percentages of original culture was observed up to E_{420} of 0.30, thereafter the linear relationship was not obeyed (Figure 13). The same suspension, when diluted to give readings below 0.30 showed linear relationship even to E_{420} of 0.8. The low reading observed in dense cultures was due to secondary scattering in the suspension which redirects light towards the receiving photocell. Lambert-Beer law does not hold at high bacterial concentration. The range of concentrations to which the law

FIGURE 13

THE EFFECT OF CELL CONCENTRATION ON THE OPTICAL DENSITY READING OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) CULTURE

◇—◇ E_{420} of diluted suspensions

□—□ Observed E_{420} of undiluted suspensions


Optical density at 420 nm.
0·8
0·6
0·4
0·2
0
20
40
60
80
100
% (v/v) of original suspension

applies depends partly on the wavelength of the incident light (Gerrard et al., 1961).

(d) The Relationship Between Optical Density And Colony Count For Vegetative Cells

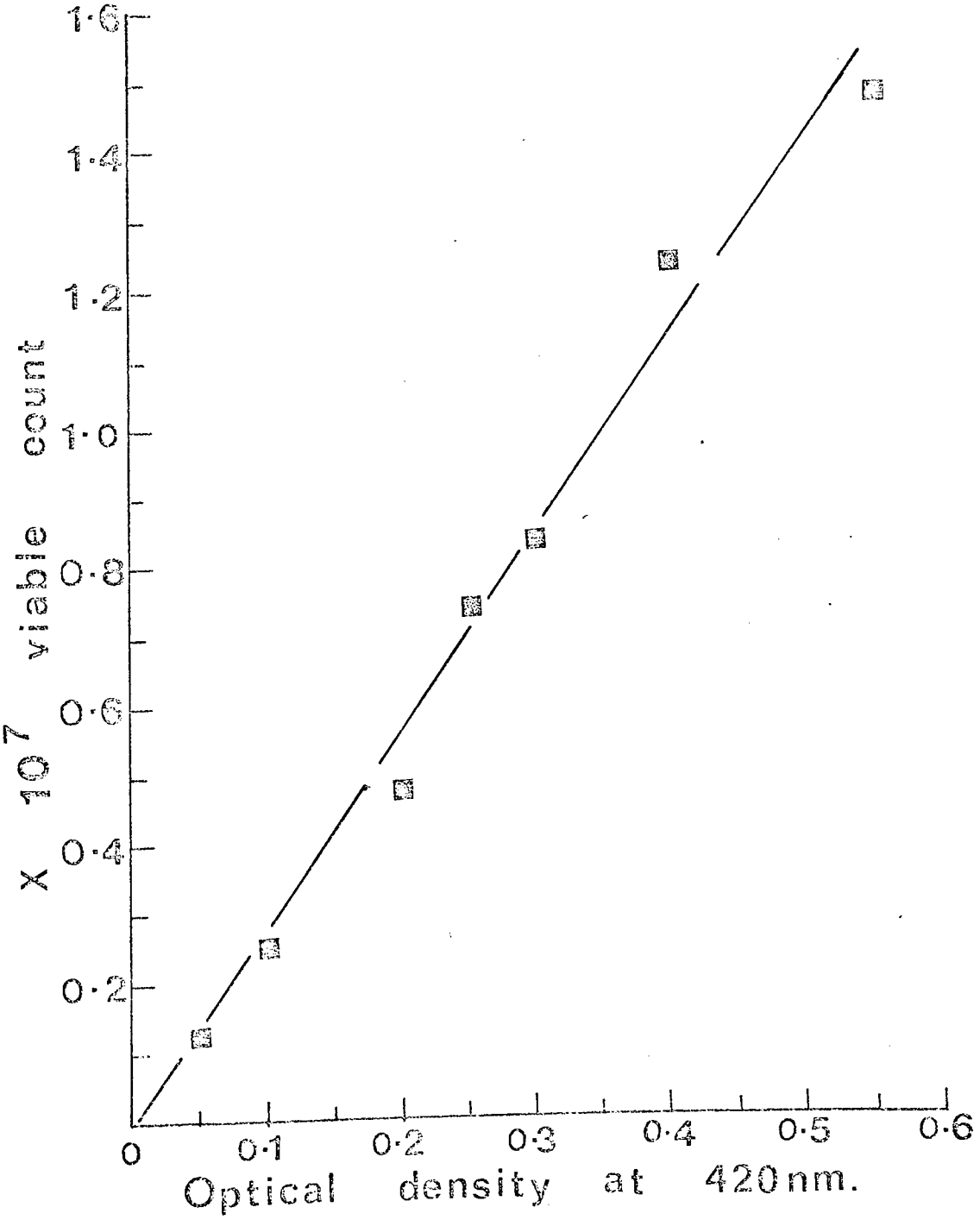
A culture of B. stearothermophilus was grown in defined medium to mid-log phase. The cells were harvested in a centrifuge and resuspended in fresh medium. The optical density was determined and sample was taken for colony count determination as described in Section 2.D. The same culture was then diluted with fresh medium to various optical densities and the colony counts again scored. Figure 14 illustrates the relationship between optical density and colony count of vegetative cells of B. stearothermophilus. A linear relationship was obtained between optical density (E_{420}) and colony count over the range of optical densities studied.

R. REPRODUCIBILITY OF B. STEAROTHERMOPHILUS GROWTH CURVE

The reproducibility of growth curves was assessed by five replicate cultures of B. stearothermophilus NCIB 8919 grown in defined medium. Growth was performed using 25 ml. of medium contained in a 100 ml. conical flask. The inoculum was 0.1 ml. log-phase cells, washed twice with pre-warmed medium by repeating centrifugation and resuspension. The optical density of the inoculum was adjusted such that when 0.1 ml. was added to 24.9 ml. of the growth medium, the optical density of the starting culture was 0.02 to 0.05.

FIGURE 14

THE RELATIONSHIP BETWEEN OPTICAL DENSITY AND VIABLE COUNT OF VEGETATIVE CELLS OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)


1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
X 10 7 viable count
0
0·1
0·2
0·3
0·4
0·5
0·6
Optical density at 420nm.

Cultures were incubated at 55°C in a Rickle reciprocal shaker operating at 110 x 3.8 cm. throws per minute. Growth was followed by withdrawing sample at suitable time intervals and the optical density determined. The sample was returned to the flask after measurement. The amount of water lost under the experimental condition was estimated to be 3% over a 9 hours incubation period at 55°C. It is unlikely that the optical density reading was drastically affected by the evaporation loss at this low level.

Doubling times were determined from the linear part of the growth curves, corresponding to log phase growth. The results are shown in Table 10. It can be seen that acceptable reproducibility was achieved.

TABLE 10

THE REPRODUCIBILITY OF DOUBLING TIME DETERMINATIONS

FLASK NUMBER	DOUBLING TIME (MINUTES)
1	63
2	60
3	66
4	66
5	60

Mean: 63

Standard deviation: ± 3

Coefficient of variation: 4.8%

3. STUDIES ON THE NUTRITIONAL REQUIREMENTS OF BACILLUS STEAROTHERMOPHILUS

A. INTRODUCTION

B. stearothermophilus species show great variability in their nutritional requirements for growth and sporulation. Campbell and Williams (1953) reported growth of most Bacillus species using glucose-mineral base medium containing 0.3% basamin (yeast autolysate). Employing the reported value of amino acid and vitamin content in basamin, they devised a synthetic medium equivalent to 0.3% basamin.

In the present study, this synthetic medium was used initially and the various components of the medium needed for growth were determined. The object was to devise a simple defined medium containing only the essential components used for growth and sporulation of B. stearothermophilus. Next, the concentration of essential nutrient components that would support growth to a desired population size were determined by separate growth depletion studies. In this method, a series of experiments to obtain data relating the extent of growth (E_{420}) to the concentration of the medium components was determined. This data would enable the formulation of media deficient in any desired component, but containing a controlled excess of all other components used for growth. The deficiency of some medium component was considered desirable for the initiation of bacterial sporulation (Grelet, 1957; Vinter, 1969).

B. EXPERIMENTAL

Table 11 illustrates the synthetic medium employed for the determination of the nutritional requirements of B. stearothermophilus. It differs from the original medium of Campbell and Williams (1953) in that sodium acetate was not used. Some preliminary investigations showed that this compound was not required for growth.

The nutritional requirements of the bacteria were determined by inoculating a series of flasks containing 24.9 ml. of the medium depleted of one medium component with 0.1 ml. of inoculum such that the optical density of the starting culture was 0.02-0.04 at E_{420}.

The inocula were prepared by inoculating a complete synthetic medium with a loopful of stock spore suspension. This, after growth overnight at 55°C, was passaged twice in the same medium and the log phase cells were harvested and washed three times with sterile water by repeating centrifugation and resuspension.

The inoculated cultures were incubated at 55°C in a Mickle reciprocal shaker as described in Section 2.R. The optical density (E_{420}) of the culture was read after 17 hours incubation and the results are shown in Table 12.

TABLE II

CHEMICALLY DEFINED MEDIUM USED IN THE DETERMINATION OF NUTRITIONAL REQUIREMENTS FOR GROWTH OF *B. STEAROTHERMOPHILUS*

(MODIFIED FROM CAMPBELL AND WILLIAMS, 1953)

CONSTITUENT	CONCENTRATION (MOLARITY)
L-Arginine	6.02×10^{-4}
L-Cystine	2.00×10^{-4}
L-Glutamic acid	6.80×10^{-4}
L-Histidine	2.90×10^{-4}
DL-Isoleucine	1.06×10^{-3}
L-Leucine	1.46×10^{-3}
L-Lysine	1.06×10^{-3}
DL-Methionine	4.02×10^{-4}
L-Tryptophan	2.90×10^{-4}
L-Valine	1.20×10^{-3}
Thiamin-HCl	4.40×10^{-7}
Riboflavin	4.00×10^{-7}
Nicotinic acid	1.20×10^{-5}
Pantothenic acid	2.10×10^{-6}
Pyridoxal	3.60×10^{-7}
Biotin	3.60×10^{-8}
Folic acid	1.40×10^{-7}
Glucose	1.00×10^{-2}
Na_2HPO_4	1.76×10^{-2}
KH_2PO_4	7.30×10^{-3}
NH_4Cl	1.87×10^{-2}
NaCl	1.70×10^{-2}
$MgCl_2$	2.40×10^{-5}
$FeCl_3$	1.80×10^{-5}
$CaCl_2$	2.20×10^{-5}

pH = 7.0-7.2

TABLE 12

THE NUTRITIONAL REQUIREMENTS FOR VEGETATIVE GROWTH* OF

B. STEAROTHERMOPHILUS SPECIES

OMITTED CONSTITUENTS \ STRAINS	NCIB 8919	NCIB 8920	NCTC 10,003 (wild type)	NCTC 10,003 (mutant)
L-Arginine	2.3	1.4	1.8	2.0
L-Cystine	2.2	1.0	1.9	1.7
L-Histidine	2.4	2.0	1.7	2.0
DL-Isoleucine	2.3	0.8	0.9	1.5
L-Leucine	0.4	1.5	0.4	1.8
L-Lysine	1.9	1.5	1.8	1.9
DL-Methionine	0.2	0.1	0	0.3
L-Tryptophan	1.6	0.2	1.8	2.0
L-Valine	0	0	0	1.8
Thiamin-HCl	0.3	0.2	0	1.7
Riboflavin	1.8	2.0	1.8	1.8
Nicotinic acid	0	0	0	1.5
Pantothenic acid	1.6	2.0	1.8	2.0
Pyridoxal	1.7	0.8	1.8	1.8
Biotin	0	0	0	1.7
Folic acid	0.9	2.0	1.6	1.8
Complete control	1.8	1.9	1.8	2.2

* Measured as optical density at E_{420}.

C. AMINO ACID AND VITAMIN REQUIREMENTS FOR GROWTH

The results presented in Table 12 are the average of at least two separate experiments. O'Brien and Campbell (1957) used the criterion that when the optical density in the deletion medium was less than one-third of that of the complete medium, the culture was considered negative with respect to growth. Using the same criterion, it can be seen that the vegetative cells of B. stearothermophilus strain NCIB 8919, NCTC 10,003 (wild) required at least L-valine, L-leucine, DL-methionine, biotin, nicotinic acid and thiamin for growth. Strain NCIB 8920 did not require L-leucine but required L-tryptophan instead. B. stearothermophilus NCTC 10,003 (mutant) did not require any of the growth factors except DL-methionine. This compound was later shown not required by this strain, if the medium was supplemented with sulphate. The DL-methionine requirement of other strains however, could not be substituted by sulphate.

D. INTERREACTION OF AMINO ACIDS REQUIRED FOR GROWTH

In an attempt to simplify the medium furthur, it was observed that if L-leucine was omitted from the medium, the cells did not show a requirement for L-valine. To test the observation, a series of flasks with media containing: (1) L-valine and L-leucine (2) L-valine alone (3) L-leucine alone and (4) no L-valine or L-leucine were prepared and growth curve for each was determined using B. stearothermophilus

NCIB 8919 as described in Section 2.R. Medium components that were shown previously non-essential for growth was excluded from these media. The results are shown in Figure 15. It is obvious that the inclusion of L-leucine in the medium necessitated the presence of L-valine and growth took place in the absence of both amino acids. Figure 16 illustrates the results of growth in fixed amount of L-leucine (1.46×10^{-3} M.) plus varying amounts of L-valine. It can be seen that valine of at least 1.9×10^{-5} M. was needed to overcome the suppression of growth by L-leucine. For optimal growth, valine of at least 7.5×10^{-5} M. was needed.

E. SOME PRELIMINARY STUDIES ON SPORULATION IN BACILLUS STEAROTHERMOPHILUS

(a) Reduction Of Glucose Concentration And The Inclusion Of Manganese In Medium

The medium devised by Campbell and Williams (1953) contained a high level of glucose (0.01 M.) and lacked manganese. Excessive amounts of carbon and energy source have been known to inhibit sporulation which also required the presence of manganese ions.

The object of the present study was to devise a medium that would give optimal growth and sporulation. Consequently, a medium with a lower concentration of glucose (1.5×10^{-3} M.) and the presence of manganese (1.0×10^{-4} M.) (concentration quoted by Anderson and Friesen, 1972) was used for subsequent studies unless stated otherwise.

FIGURE 15

GROWTH CURVES OF B. STEAROTHERMOPHILUS NCIB 8919 IN MEDIA CONTAINING VARIOUS COMBINATIONS OF L-LEUCINE AND L-VALINE

+Leucine = 1.46×10^{-3} Molar

+Valine = 1.20×10^{-3} Molar

Note: Time scale displaced 1 hr. for each curve.

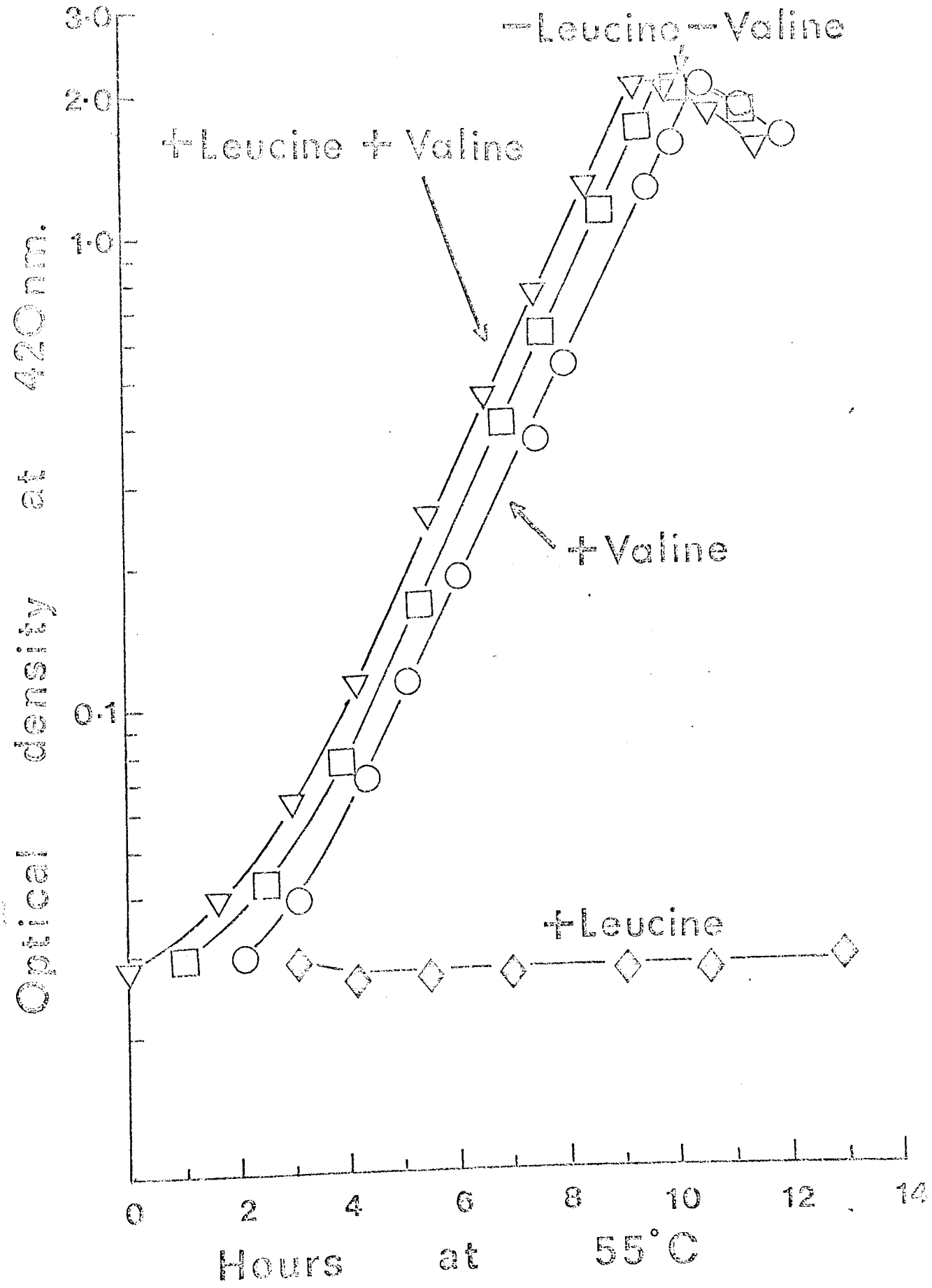

−Leucine −Valine
+Leucine +Valine
+Valine
+Leucine
Optical density at 420 mμ.
3·0
2·0
1·0
0·1
0
2
4
6
8
10
12
14
Hours at 55°C

FIGURE 16

GROWTH CURVES OF B. STEAROTHERMOPHILUS NCIB 8919 IN MEDIA CONTAINING A FIXED CONCENTRATION OF L-LEUCINE (1.46×10^{-3} M.) AND GRADED CONCENTRATIONS OF L-VALINE IN MOLAR.

Blank

8.8×10^{-7} M.

1.9×10^{-5} M.

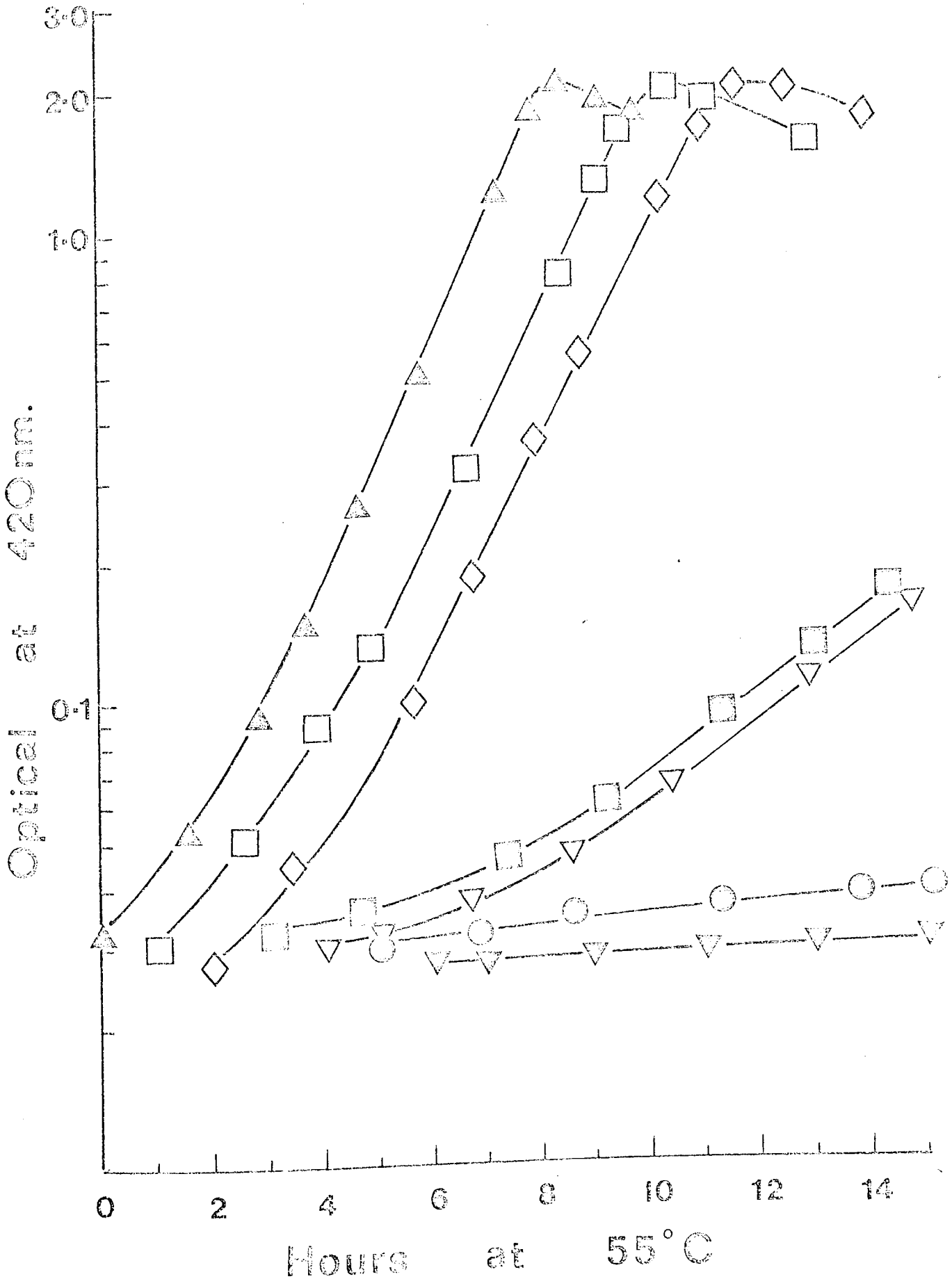
5.6×10^{-5} M.

7.5×10^{-5} M.

1.5×10^{-4} M.

3.0×10^{-4} M.

Note: Time scale displaced 1 hr. for each curve.


3·0
2·0
1·0
0·1
Optical at 420 nm.
0
2
4
6
8
10
12
14
Hours at 55° C

It was observed that following the reduction of glucose concentration and the addition of manganese to the medium, some sporulation occurred in B. stearothermophilus NCIB 8919, NCTC 10,003 (wild type), NCIB 8920 (20-25%) and also 3-5% in NCTC 10,003 (mutant) following overnight growth (15 hours).

(b) The Addition Of L-Glutamic Acid

Anderson and Friesen (1972) found sporulation of B. stearothermophilus in chemically defined medium improved following the addition of L-glutamic acid. In the present study, L-glutamic acid at a concentration of 2.4×10^{-3} M. was added to the medium and the effect on sporulation studied. It was observed that the addition of L-glutamic acid greatly promoted the sporulation of B. stearothermophilus NCTC 10,003 (mutant) to about 36% but was without effect on other strains studied.

F. THE FORMULATION OF CHEMICALLY DEFINED MEDIUM FOR GROWTH AND SPORULATION OF B. STEAROTHERMOPHILUS

Using the data obtained, a chemically defined medium was formulated which contained growth factors biotin, nicotinic acid, thiamin and DL-methionine. Table 13 shows the chemical composition of the medium. This medium supported good growth and sporulation (20-25%) of vegetative cells of B. stearothermophilus NCIB 8919, and NCTC 10,003 (wild).

For the optimal growth of strain NCIB 8920, L-tryptophan

TABLE 13

CHEMICALLY DEFINED MEDIUM FOR THE GROWTH AND SPORULATION OF VEGETATIVE CELLS* OF B. STEAROTHERMOPHILUS STRAINS NCIB 8919, NCIB 8920 AND NCTC 10,003 (WILD TYPE)

CONSTITUENT	CONCENTRATION (MOLARITY)
Na_2HPO_4	1.76×10^{-2}
KH_2PO_4	7.30×10^{-3}
NH_4Cl	1.87×10^{-2}
$MgCl_2.6H_2O$	2.40×10^{-5}
$FeCl_2.4H_2O$	1.80×10^{-5}
$CaCl_2.6H_2O$	2.20×10^{-5}
$MnSO_4.4H_2O$	1.00×10^{-4}
DL-Methionine	4.02×10^{-4}
L-Tryptophan**	2.90×10^{-4}
Thiamin-HCl	4.40×10^{-7}
Nicotinic acid	1.20×10^{-5}
Biotin	3.60×10^{-8}
Glucose	1.50×10^{-3}

pH 7.0-7.2

*When growth was initiated from spores, 7.50×10^{-5} M. valine was included in the medium.

**Not required for strains NCIB 8919, NCTC 10,003 (wild type).

at a concentration of 2.9×10^{-4} M. was included.

When growth was initiated from spores, it was found that the inclusion of 7.5×10^{-5} M. of L-valine was essential to all strains studied except strain NCTC 10,003 (mutant), to overcome the long lag exhibited by culture omitting this amino acid.

B. stearothermophilus NCTC 10,003 (mutant) did not require any of the amino acids and vitamins studied. Spores germination and outgrowth as well as vegetative cell growth occurred in the absence of any growth factor. A medium modified from that previously used by Anderson and Friesen (1972) for growth of this organism was formulated (Table 14). This medium gave good growth and sporulation of the mutant at incubation temperature of 55°C or 60°C.

TABLE 14

CHEMICALLY DEFINED MEDIUM FOR THE GROWTH AND SPORULATION OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

CONSTITUENT	CONCENTRATION (MUTANT)
Na_2HPO_4	1.76×10^{-2}
KH_2PO_4	7.30×10^{-3}
NH_4Cl	9.35×10^{-3}
$MgSO_4.7H_2O$	5.00×10^{-4}
$FeSO_4.7H_2O$	1.00×10^{-5}
$MnSO_4.4H_2O$	1.00×10^{-4}
$CaCl_2.6H_2O$	1.00×10^{-4}
L-Glutamic acid	2.40×10^{-3}
Glucose	1.50×10^{-3}

pH 7.0-7.2

4. GROWTH DEPLETION STUDIES ON BACILLUS STEAROTHERMOPHILUS IN DEFINED MEDIA

A. STUDIES ON BACILLUS STEAROTHERMOPHILUS NCIB 8919

(a) Experimental

Table 15 illustrates the composition of the media used in growth depletion studies. Wherepossible, the molarity of ions used was kept as constant as possible from one study to another. This necessitated the use of NaCl or Na_2SO_4 in some cases.

Growth was performed using 25 ml. of the medium graded in the concentrations of depleting component. The inoculum was 0.1 ml. of log phase cells, grown in a medium containing a depleting concentration of the medium component under study and washed twice with prewarmed medium deficient in the depleting component and finally resuspended in the same medium. The optical density of the medium was adjusted such that when 0.1 ml. was added to 24.9 ml. of the growth medium, the optical density of the starting culture was 0.02 to 0.04. Cultures were incubated at 55°C in a Mickle reciprocal shaker. Growth was followed by optical density measurement as described in Section 2.R.

(b) Glucose depletion

The growth curves of cultures with graded concentrations of glucose are shown in Figure 17. Growth ceased abruptly

TABLE 15

COMPOSITION OF MEDIA USED IN GROWTH DEPLETION STUDIES OF B. STEAROTHERMOPHILUS NCIB 8919

Depletion → / Medium component ↓	Glucose	Nitrogen	Biotin	Nicotinic acid	Thiamin	Methionine	Magnesium	Iron	Calcium	Manganese
Na_2HPO_4	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}
KH_2PO_4	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}
NH_4Cl	1.87×10^{-2}	*	1.87×10^{-2}	1.87×10^{-2}	1.87×10^{-2}	1.87×10^{-2}	1.87×10^{-2}	1.87×10^{-2}	1.87×10^{-2}	1.87×10^{-2}
$MgCl_2.6H_2O$	2.40×10^{-5}	2.40×10^{-5}	2.40×10^{-5}	2.40×10^{-5}	2.40×10^{-5}	2.40×10^{-5}	*	2.40×10^{-5}	2.40×10^{-5}	2.40×10^{-5}
$FeCl_2.4H_2O$	1.80×10^{-5}	1.80×10^{-5}	1.80×10^{-5}	1.80×10^{-5}	1.80×10^{-5}	1.80×10^{-5}	1.80×10^{-5}	*	1.80×10^{-5}	1.80×10^{-5}
$CaCl_2.6H_2O$	2.20×10^{-5}	2.20×10^{-5}	2.20×10^{-5}	2.20×10^{-5}	2.20×10^{-5}	2.20×10^{-5}	2.20×10^{-5}	2.20×10^{-5}	*	2.20×10^{-5}
$MnSO_4.4H_2O$	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	*
Biotin	3.60×10^{-8}	3.60×10^{-8}	*	3.60×10^{-8}	3.60×10^{-8}	3.60×10^{-8}	3.60×10^{-8}	3.60×10^{-8}	3.60×10^{-8}	3.60×10^{-8}
Nicotinic acid	1.20×10^{-5}	1.20×10^{-5}	1.20×10^{-5}	*	1.20×10^{-5}	1.20×10^{-5}	1.20×10^{-5}	1.20×10^{-5}	1.20×10^{-5}	1.20×10^{-5}
Thiamin-HCl	4.40×10^{-7}	4.40×10^{-7}	4.40×10^{-7}	4.40×10^{-7}	*	4.40×10^{-7}	4.40×10^{-7}	4.40×10^{-7}	4.40×10^{-7}	4.40×10^{-7}
DL-Methionine	4.02×10^{-4}	4.02×10^{-4}	4.02×10^{-4}	4.02×10^{-4}	4.02×10^{-4}	*	4.02×10^{-4}	4.02×10^{-4}	4.02×10^{-4}	4.02×10^{-4}
Glucose	*	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Na_2SO_4										1.00×10^{-4}
NaCl		1.87×10^{-2}								

* Component graded.

All concentrations expressed as molarities pH 7.0-7.2 in each case.

FIGURE 17

GROWTH CURVES OF GLUCOSE DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCIB 8919

All concentrations in molar.

○—○ 1.0×10^{-3} M.

△—△ 3.0×10^{-3} M.

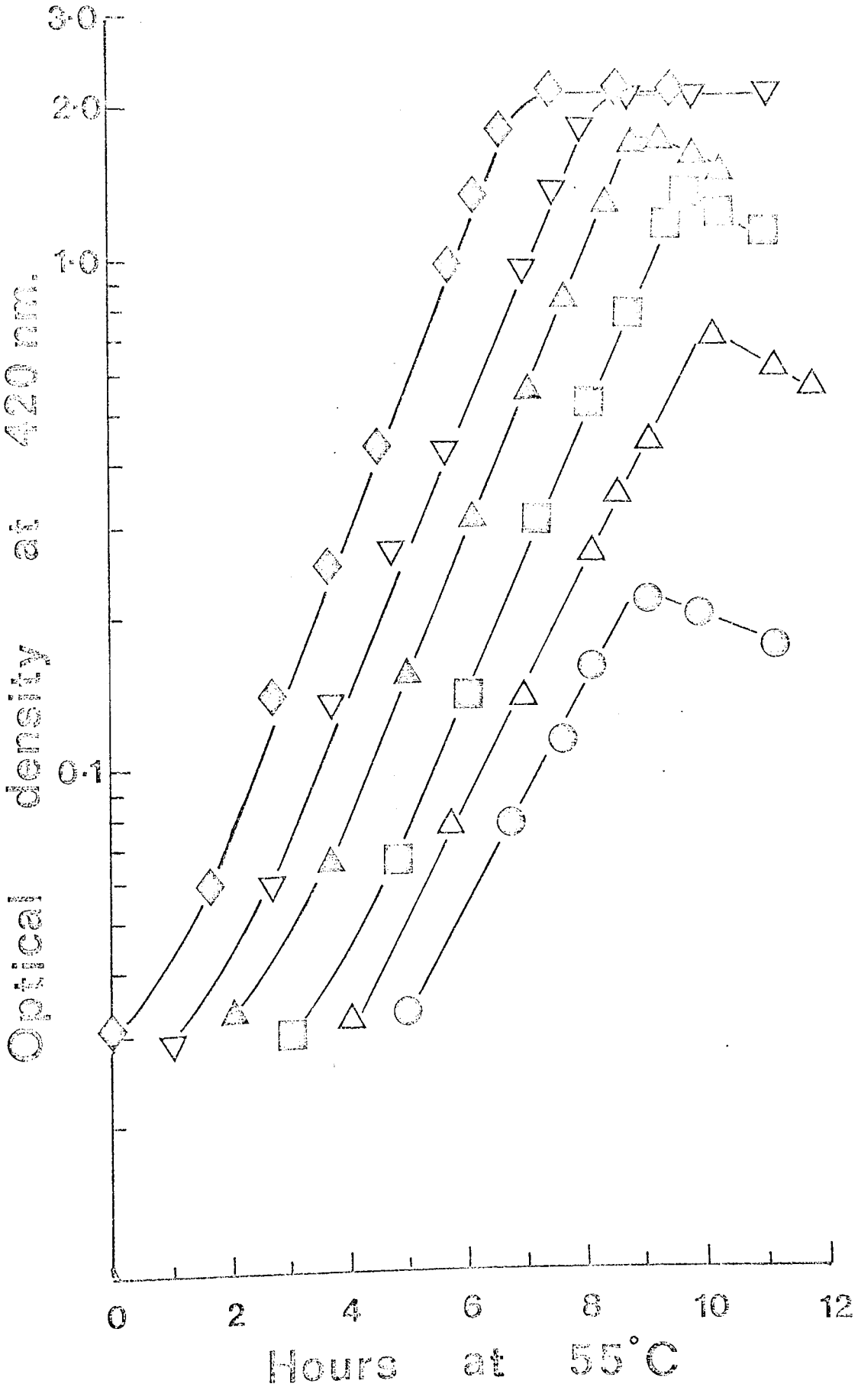
□—□ 6.0×10^{-3} M.

△—△ 8.0×10^{-3} M.

▽—▽ 9.5×10^{-3} M.

◇—◇ 1.2×10^{-2} M.

Note: Time scale displaced 1 hr. for each curve.


3·0
2·0
1·0
0·1
Optical density at 420 mμ.
0
2
4
6
8
10
12
Hours at 55°C

as soon as the culture was depleted of glucose. The optical density fell slightly on further incubation showing that some lysis of cells might have occurred. The possibility that the fall in optical density is due to cell size changes on the depletion of oxygen cannot be ruled out.

The maximum growth possible under the experimental conditions was E_{420} of 2.2. Cultures with glucose in excess of that required for growth did not show any fall in optical density as opposed to cultures depleted of glucose, showing the importance of energy level under conditions of probable oxygen depletion, on the prevention of cell lysis or cell size changes.

When E_{420} maximum values taken from the growth curves were plotted against the initial glucose concentrations, a linear relationship was obtained below a critical concentration (Figure 18). Abovewhich, some other experimental limitations such as the degree of aeration, trace elements availability etc. limited further increase in growth. Since the straight line passed through the origin, it was concluded that the bacteria used glucose as sole carbon/energy source, other carbon containing compounds present in the medium (vitamins, methionine) were not used for this same purpose.

(c) Methionine Depletion

Figure 19 illustrates the growth curves of cultures with various initial concentrations of methionine. Unlike glucose

FIGURE 18

THE RELATIONSHIP BETWEEN E_{420} (MAXIMUM) AND DIFFERENT INITIAL CONCENTRATIONS OF GLUCOSE OR METHIONINE USED IN THE CULTIVATION OF B. STEAROTHERMOPHILUS NCIB 8919

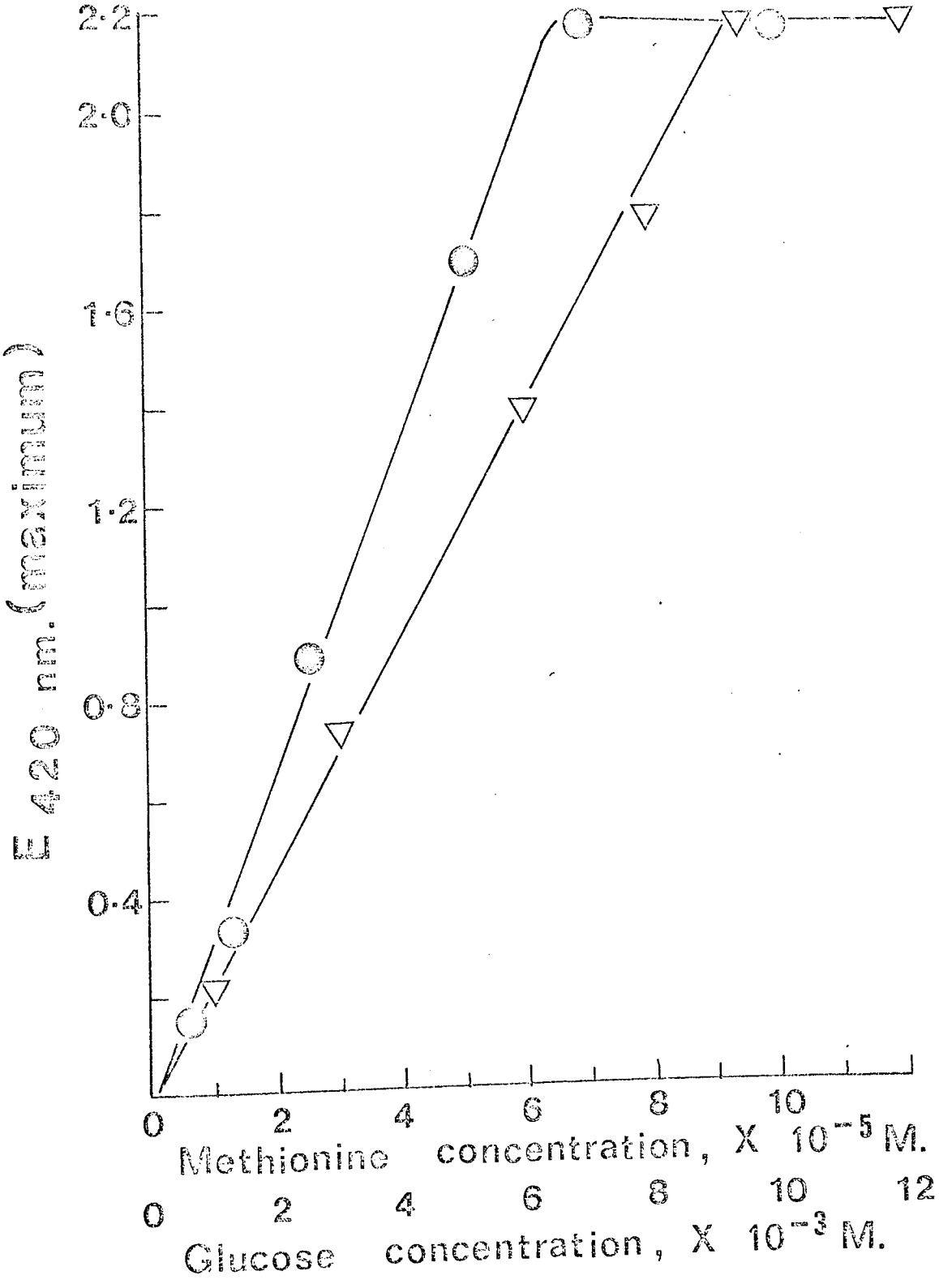

E 420 mμ (maximum)
2·2
2·0
1·6
1·2
0·8
0·4
0
2
4
6
8
10
Methionine concentration, X 10⁻⁵ M.
0
2
4
6
8
10
12
Glucose concentration, X 10⁻³ M.

FIGURE 19

GROWTH CURVES OF METHIONINE DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCIB 8919

All concentrations in molar.

▽—▽ 6.3×10^{-6} M.

◇—◇ 1.2×10^{-5} M.

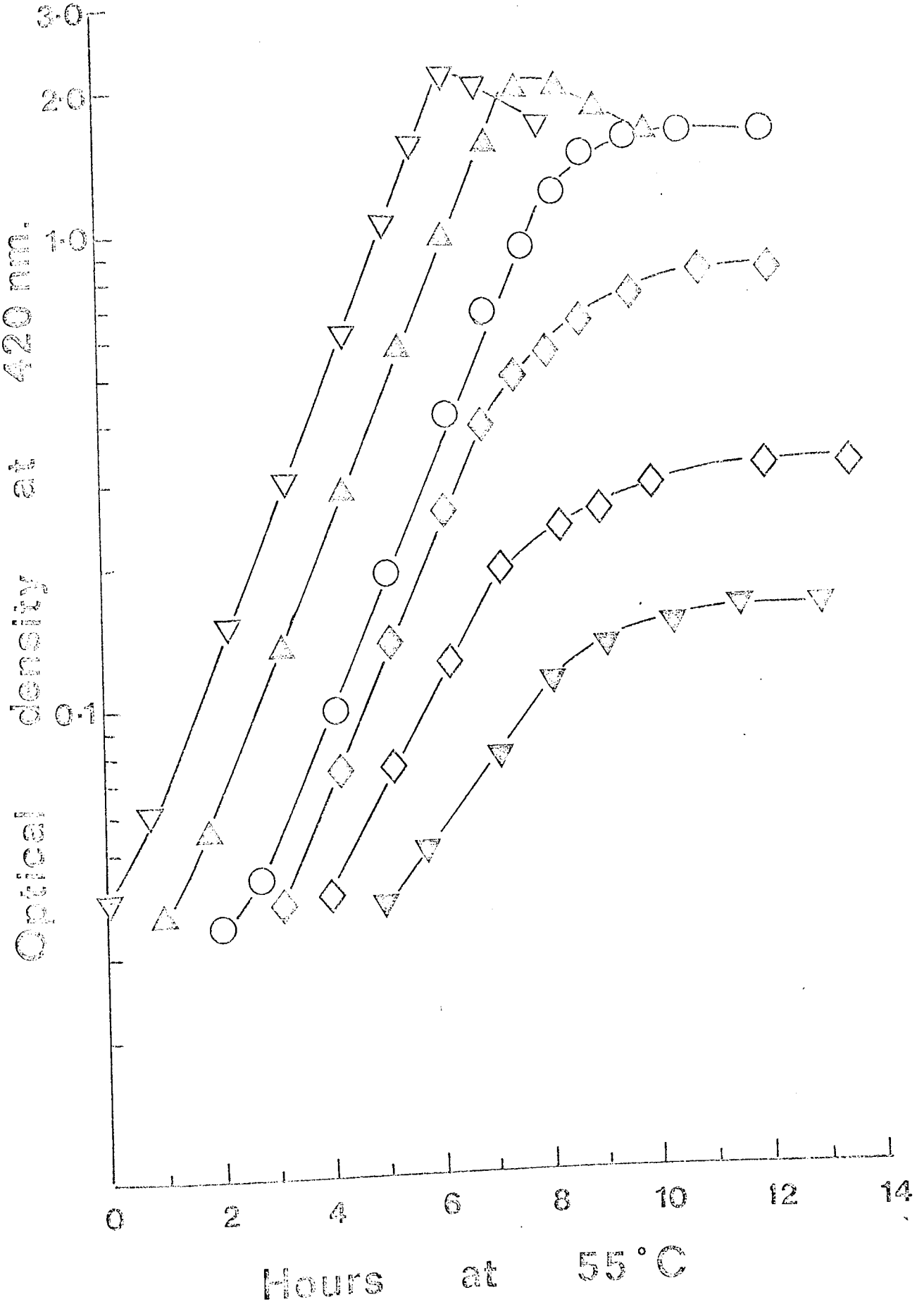
◆—◆ 2.5×10^{-5} M.

○—○ 5.0×10^{-5} M.

▲—▲ 7.0×10^{-5} M.

▽—▽ 1.0×10^{-4} M.

Note: Time scale displaced 1 hr. for each curve.


3·0
2·0
1·0
0·1
Optical density at 420 mμ.
0
2
4
6
8
10
12
14
Hours at 55°C

depleted growth, the onset of depletion after logarithmic growth was not sharp. The growth rate of the culture progressively fell to zero over a 3-4 hours period after the onset of depletion.

When E_{420} maximum values taken from the growth curves were plotted against the initial methionine concentrations, a linear relationship was obtained below a critical concentration (Figure 18). The maximum growth achieved was similar to that of glucose depleted cultures showing that some common factors limited further growth of both types of cultures.

(d) Vitamin Depletion

Figures 20, 21 and 22 illustrate the growth curves of cultures with various initial concentrations of biotin, nicotinic acid or thiamin in the media.

The response of cultures following vitamin depletion was similar in each case. The onset of depletion was not sharp, growth rate of the culture fell progressively after logarithmic growth. The fall of growth rate was faster following nicotinic acid and thiamin depletions than was with biotin depletion.

When E_{420} taken from the growth curves at the onset of depletion were plotted against the initial concentrations of vitamin used, linear relationship was obtained below a certain critical concentration (Figure 23).

Since all the lines did not pass through the origin, it is

FIGURE 20

GROWTH CURVES OF BIOTIN DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCIB 8919

All concentrations in molar

◆—◆ 1.10×10^{-10} M.

□—□ 3.10×10^{-10} M.

▼—▼ 4.65×10^{-10} M.

◇—◇ 1.25×10^{-9} M.

●—● 1.50×10^{-9} M.

△—△ 2.10×10^{-9} M.

Note: Time scale displaced 1 hr. for each curve.

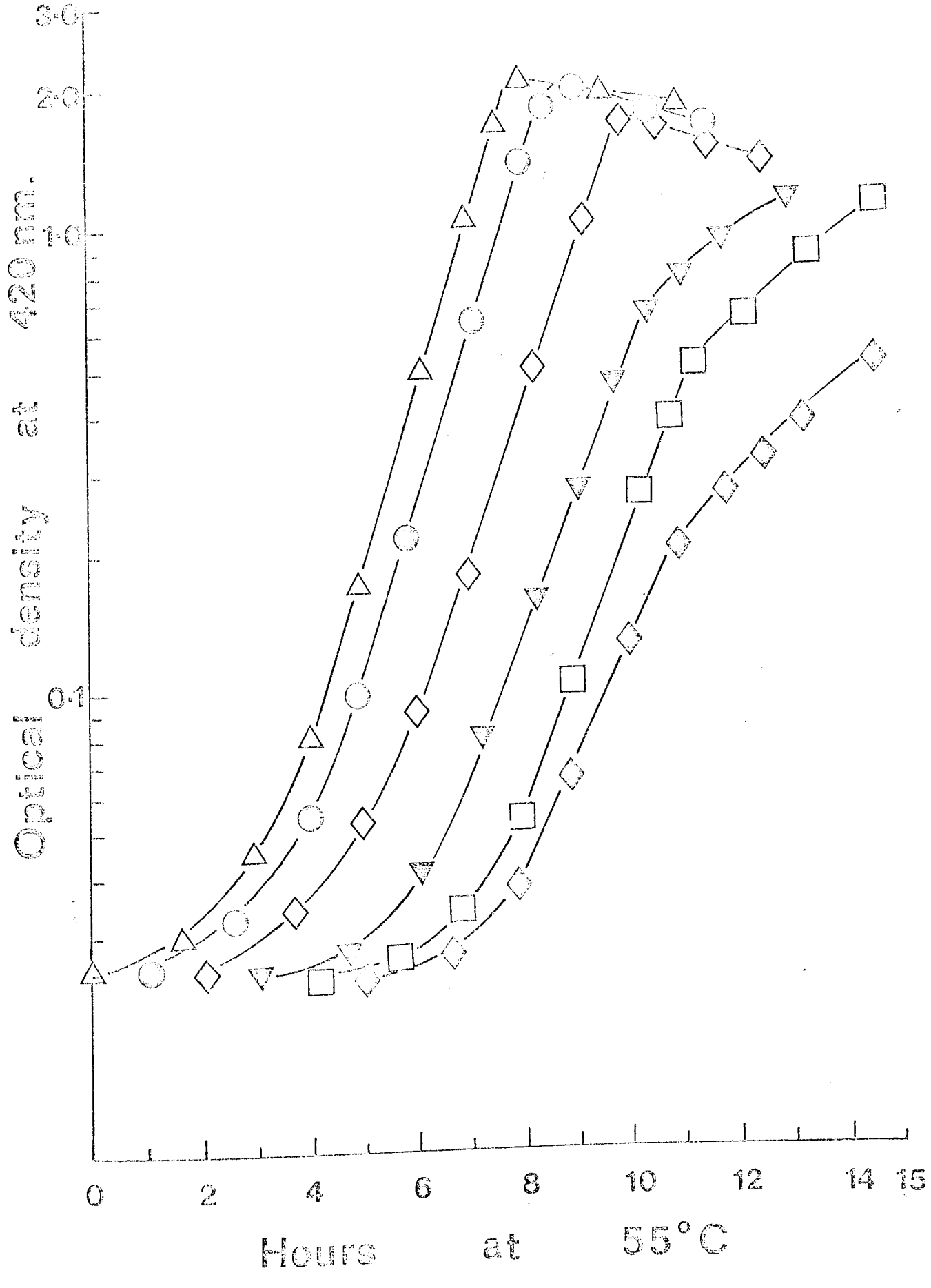

3·0
2·0
1·0
0·1
Optical density at 420 m.
0
2
4
6
8
10
12
14
15
Hours at 55° C

FIGURE 21

GROWTH CURVES OF NICOTINIC ACID DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCIB 8919

All concentrations in molar

◆—◆ 1.87×10^{-7} M.

▲—▲ 6.00×10^{-7} M.

◇—◇ 7.50×10^{-7} M.

■—■ 1.05×10^{-6} M.

△—△ 1.35×10^{-6} M.

○—○ 1.80×10^{-6} M.

Note: Time scale displaced 1 hr. for each curve.

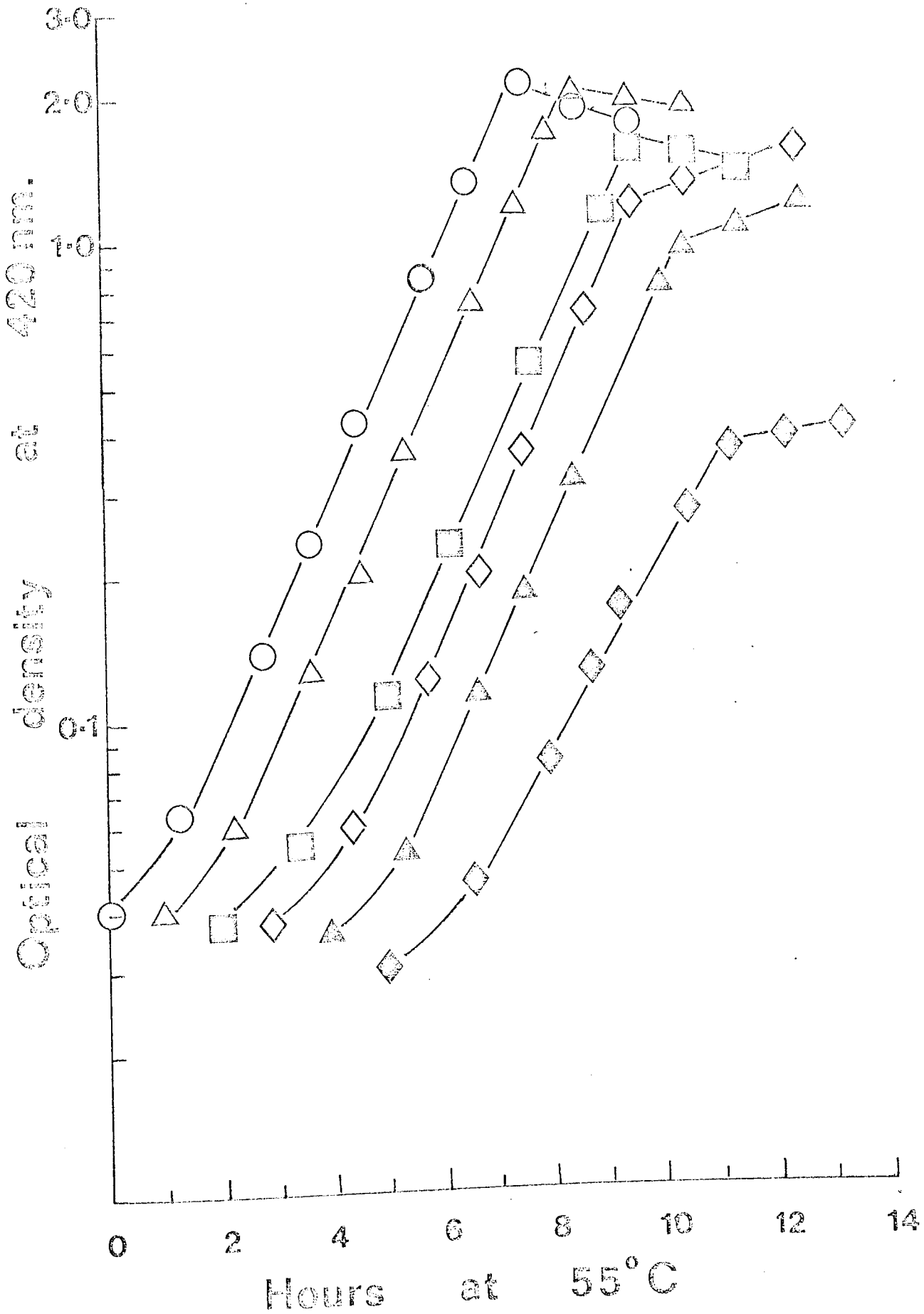

3·0
2·0
1·0
0·1
Optical density at 420 mμ.
0
2
4
6
8
10
12
14
Hours at 55°C

FIGURE 22

GROWTH CURVES OF THIAMIN DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCIB 8919

All concentrations in molar

◇—◇ 3.50×10^{-9} M.

●—● 1.30×10^{-8} M.

■—■ 3.00×10^{-8} M.

▽—▽ 4.00×10^{-8} M.

▲—▲ 5.75×10^{-8} M.

□—□ 6.75×10^{-8} M.

Note: Time scale displaced 1 hr. for each curve.

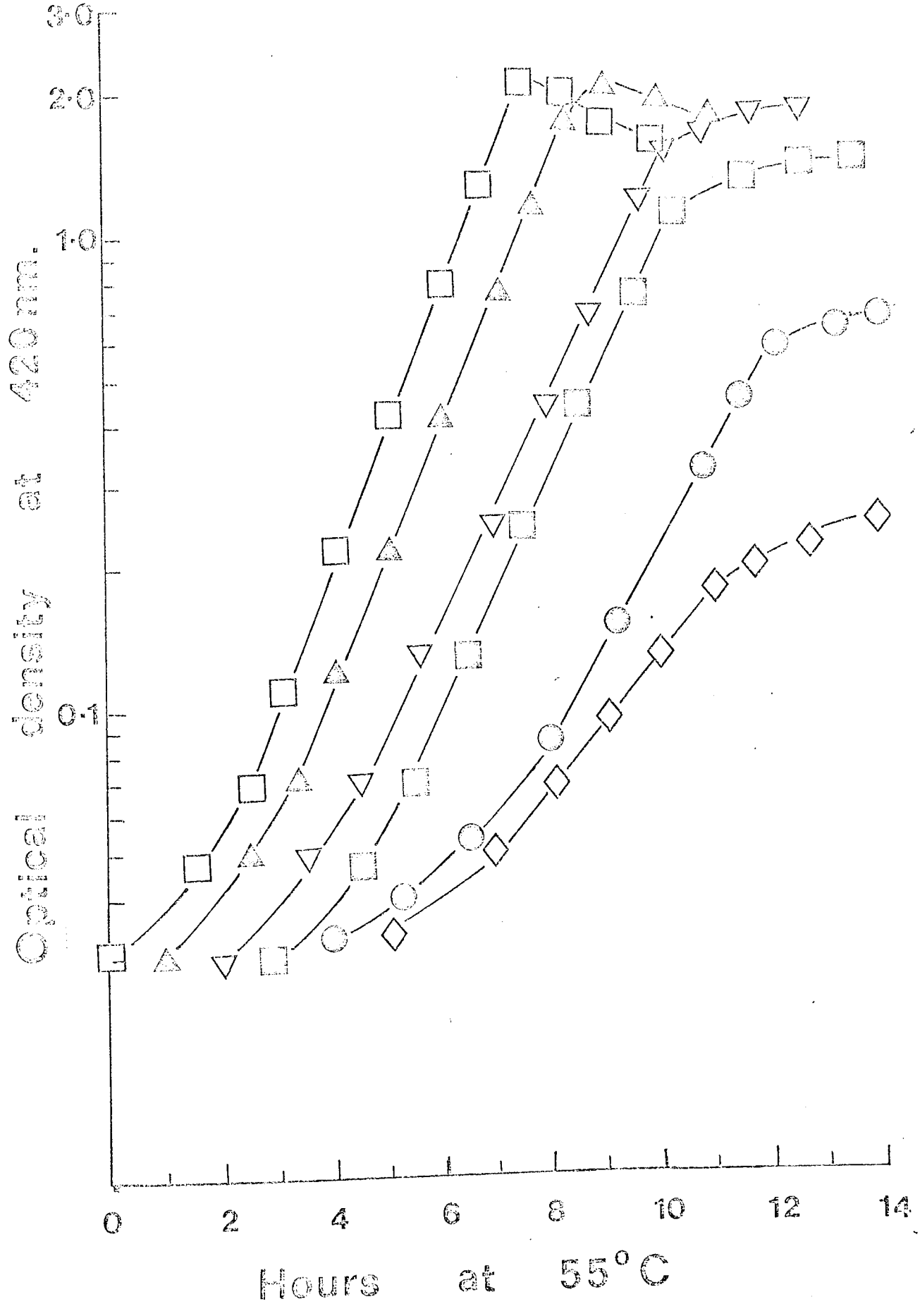

3·0
2·0
1·0
0·1
Optical density at 420 nm.
0
2
4
6
8
10
12
14
Hours at 55° C

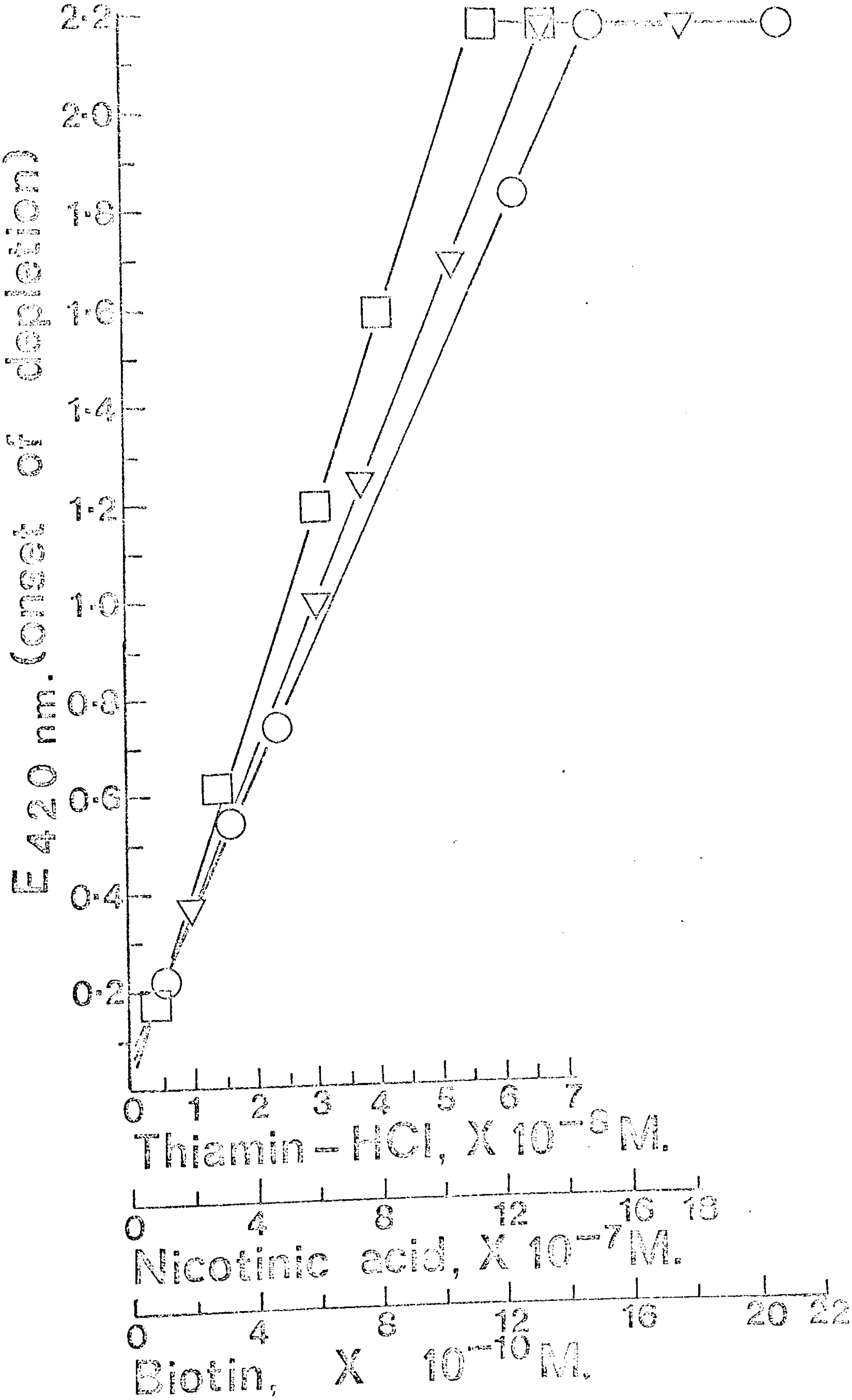
FIGURE 23

THE RELATIONSHIP BETWEEN E_{420} (ONSET OF DEPLETION) AND DIFFERENT INITIAL CONCENTRATIONS OF VITAMIN USED IN THE CULTIVATION OF B. STEAROTHERMOPHILUS NCIB 8919

▽—▽ Nicotinic acid

○—○ Biotin

□—□ Thiamin-HCl



E 420 nm. (onset of depletion)
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0 1 2 3 4 5 6 7
Thiamin – HCl, X 10⁻⁸ M.
0 4 8 12 16 18
Nicotinic acid, X 10⁻⁷ M.
0 4 8 12 16 20 22
Biotin, X 10⁻¹⁰ M.

highly probable that some slight contaminating levels of vitamin were present in the media. This might come from the inocula which had been cultivated previously in medium containing these vitamins.

The extreme sensitivity of the culture to a very small amount of vitamin could allow the detection and perhaps quantification of these vitamins. The culture was sensitive to 10^{-10} M. biotin; 10^{-8} M. thiamin and 10^{-7} M. nicotinic acids.

(e) Nitrogen Depletion

Growth curves of nitrogen depleted cultures are shown in Figure 24. Like glucose depleted cultures, the onset of depletion was followed by the immediate cessation of growth. However, nitrogen depleted cultures did not result in cell lysis.

The plot of E_{420} maximum values against the initial concentrations of NH_4Cl showed a linear relatioship below a critical concentration (Figure 25). Since the line did not pass through the origin, a contaminating level (equivalent to about 1.0×10^{-4} M. NH_4Cl) of nitrogen containing compound was present in the medium. The possibility that the other nitrogen containing compounds present in the medium (methionine and vitamins) contributed to the contaminating level of growth seems remote. Methionine was present in far greater concentrations (4.02×10^{-4} M.) than the observed contaminating level of 1.0×10^{-4} M. NH_4Cl equivalent. The contribution from the vitamins (biotin, nicotinic acid and thiamin) could not be ruled

FIGURE 24

GROWTH CURVES OF NITROGEN DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCIB 8919

All concentrations in molar.

◆—◆ 4.96×10^{-4} M.

○—○ 1.46×10^{-3} M.

▲—▲ 2.45×10^{-3} M.

■—■ 3.50×10^{-3} M.

◇—◇ 5.00×10^{-3} M.

△—△ 1.00×10^{-2} M.

Note: Time scale displaced 1 hr. for each curve.

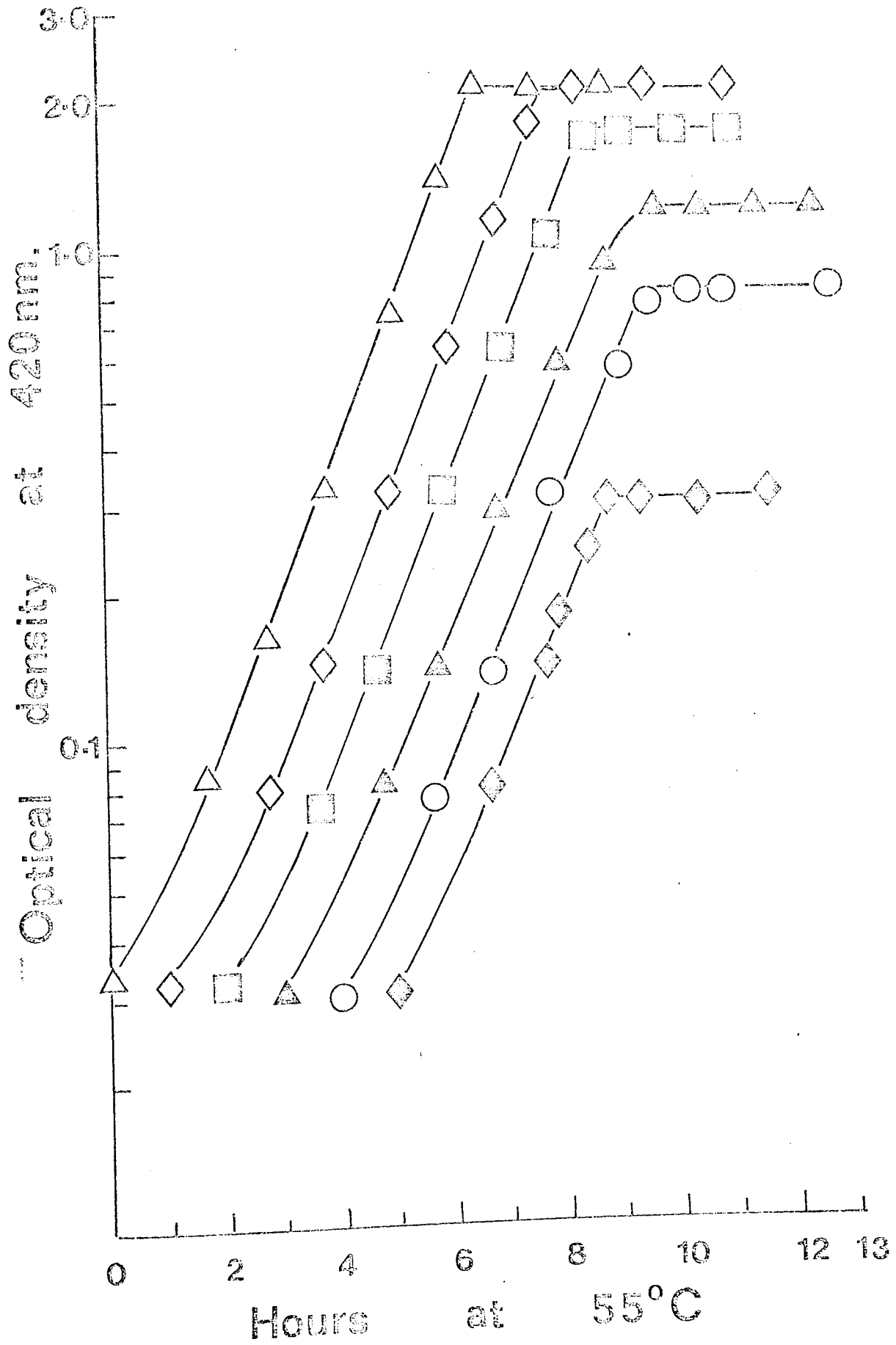
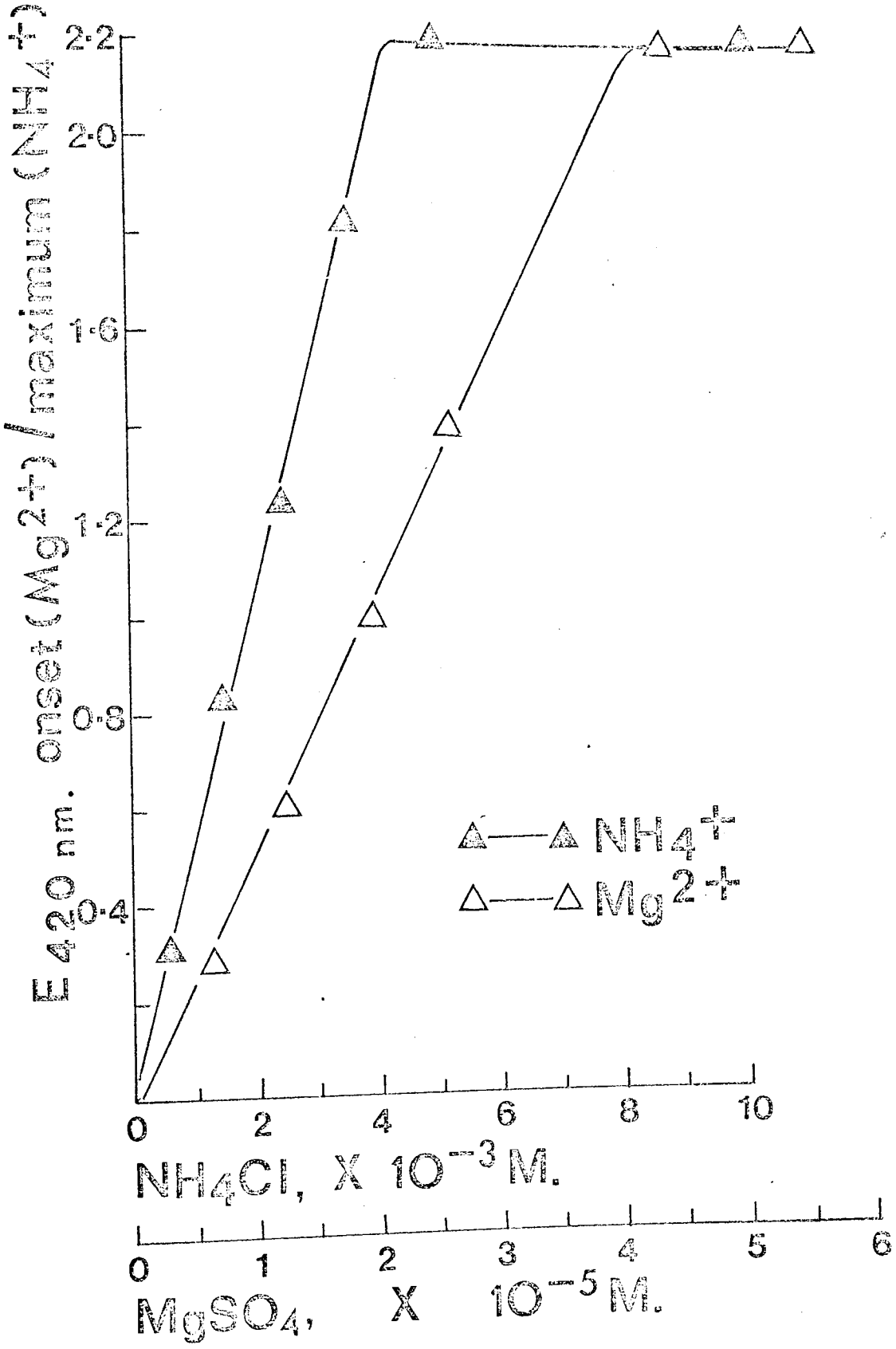

3·0
2·0
1·0
0·1
Optical density at 420 nm.
0
2
4
6
8
10
12
13
Hours at 55°C

FIGURE 25

THE RELATIONSHIP BETWEEN E_{420} (MAXIMUM/ONSET OF DEPLETION) AND DIFFERENT INITIAL CONCENTRATIONS OF NH_4Cl/MAGNESIUM USED IN THE CULTIVATION OF B. STEAROTHERMOPHILUS NCIB 8919


E 420 nm. onset (Mg2+) / maximum (NH4+)
2·2
2·0
1·6
1·2
0·8
0·4
0
2
4
6
8
10
NH4Cl, X 10⁻³ M.
0
1
2
3
4
5
6
MgSO4, X 10⁻⁵ M.
NH4+
Mg2+

out, but seems unlikely because the total nitrogen contribution from these vitamins would be less than the observed level of contamination. However, the possibility that different nitrogen containing compounds with equivalent amount of nitrogen, might support growth to different optical densities could not be ruled out. It is likely that only NH_4^+ was utilised as nitrogen source. The contaminating NH_4^+ might come from other chemical components present in the medium, since most chemicals are known not to be totally free of NH_4^+, even at AnalaR grade.

(f) Magnesium Depletion

Figure 26 illustrates the growth curves of magnesium depleted cultures. The onset of depletion was characterised by a progressively decreasing growth rate similar to vitamin depleted culture.

When E_{420} onset of depletion was plotted against the initial concentration of magnesium used, a linear relationship was obtained below a critical concentration (Figure 25). The straight line passed through the origin, showing no contamination of the medium of any utilizable magnesium.

(g) Iron, Calcium and Manganese Depletion

Cultures omitted of iron, calcium or manganese showed no reduction in maximum growth. It was concluded that the requirements for calcium, iron and manganese were low and were satisfied by the level of impurities present in other medium components.

FIGURE 26

GROWTH CURVES OF MAGNESIUM DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCIB 8919

All concentrations in molar.

△—△ 6.50×10^{-6} M.

◇—◇ 1.30×10^{-5} M.

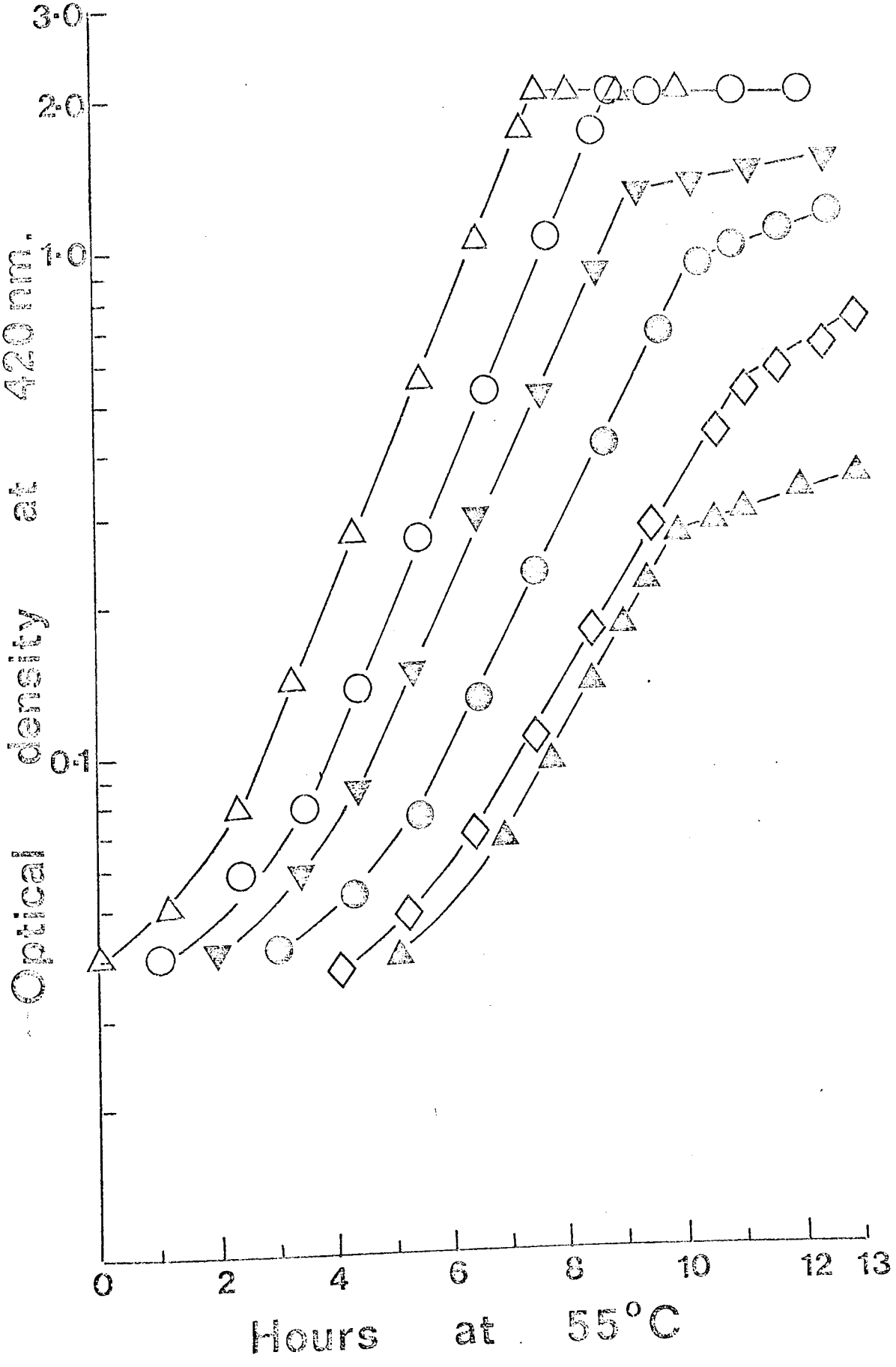
●—● 1.95×10^{-5} M.

▽—▽ 2.93×10^{-5} M.

○—○ 4.40×10^{-5} M.

△—△ 5.50×10^{-5} M.

Note: Time scale displaced 1 hr. for each curve.


3·0
2·0
1·0
0·1
Optical density at 420 mμ.
0
2
4
6
8
10
12
13
Hours at 55°C

(h) Microscopic Examination Of Cultures Under Different Nutrient Depletions

With the exception of magnesium, manganese and vitamin depleted cultures, all other cultures gave some degree of sporulation (20-25%). The spores formed under various nutrient depleted conditions appeared similar in size and shape and this aspect was not investigated further.

B. STUDIES ON BACILLUS STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

(a) Experimental

Table 16 illustrates the composition of media used for growth depletion studies of B. stearothermophilus NCTC 10,003 (mutant). Preliminary studies disclosed that glutamic acid was used both as carbon and nitrogen sources. This contribution to overall carbon and nitrogen sources was estimated by separate depletion experiments.

The contribution of glutamic acid to overall carbon needs was assessed by a growth depletion study using media containing a fixed concentration of glucose (1.0×10^{-3} M.) and varying concentrations of glutamic acid. E_{420} maximum value was determined as described before. Likewise, the contribution of glutamic acid to the overall nitrogen need was assessed by a separate growth depletion experiment using media containing a fixed concentration of NH_4Cl (1.87×10^{-3} M.) and varying concentrations of glutamic acid.

TABLE 16

COMPOSITION OF MEDIA USED IN GROWTH DEPLETION STUDIES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

Depletion → / Medium component ↓	Carbon		Nitrogen		Magnesium	Sulphate	Phosphate	Calcium	Manganese	Iron
	Glucose	L-Glutamic acid	NH_4Cl	L-Glutamic acid						
Na_2HPO_4	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}		1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}
KH_2PO_4	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}	*	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}
NH_4Cl	9.35×10^{-3}	9.35×10^{-3}	*	1.87×10^{-3}	9.35×10^{-3}	9.35×10^{-3}	9.35×10^{-3}	9.35×10^{-3}	9.35×10^{-3}	9.35×10^{-3}
$MgSO_4.7H_2O$	5.00×10^{-4}	5.00×10^{-4}	5.00×10^{-4}	5.00×10^{-4}	*		5.00×10^{-4}	5.00×10^{-4}	5.00×10^{-4}	5.00×10^{-4}
$FeSO_4.7H_2O$	1.00×10^{-5}	1.00×10^{-5}	1.00×10^{-5}	1.00×10^{-5}	1.00×10^{-5}		1.00×10^{-5}	1.00×10^{-5}	1.00×10^{-5}	*
$MnSO_4.4H_2O$	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}		1.00×10^{-4}	1.00×10^{-4}	*	1.00×10^{-4}
$CaCl_2.6H_2O$	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	*	1.00×10^{-4}	1.00×10^{-4}
Glucose	*	1.00×10^{-3}	7.50×10^{-3}	7.50×10^{-3}	7.50×10^{-3}	7.50×10^{-3}	7.50×10^{-3}	7.50×10^{-3}	7.50×10^{-3}	7.50×10^{-3}
L-Glutamic acid		*		*	2.40×10^{-3}	2.40×10^{-3}	2.40×10^{-3}	2.40×10^{-3}	2.40×10^{-3}	2.40×10^{-3}
HEPES							0·03			
Na_2SO_4					5.00×10^{-4}	*			1.00×10^{-4}	1.00×10^{-5}
KCl							7.30×10^{-3}			
$MgCl.6H_2O$						5.00×10^{-4}				
$FeCl_2.4H_2O$						1.00×10^{-5}				
$MnCl_2.4H_2O$						1.00×10^{-4}				
NaCl							1.76×10^{-2}	1.00×10^{-4}		

* Component graded.

All concentrations expressed as molarities pH 7.0-7.2 in each case.

Bacteria were grown as 25 ml. culture in 100 ml. conical flask incubated at 60°C, using reciprocal shaker as described in Section 2.R.

(b) Carbon Depletion Using Glucose

Figure 27 shows the growth curves of cultures using glucose as sole carbon source. These curves are similar in shape to those obtained with B. stearothermophilus NCIB 8919 (Figure 17).

When E_{420} maximum values taken from the curves were plotted against the initial glucose concentrations used, a linear relationship was obtained below a certain critical concentration (Figure 28). The fact that the line did not pass through the origin indicates some degrees of contamination (equivalent to about 1.0×10^{-4} M. glucose) of utilizable carbon source in the medium.

(c) Nitrogen Depletion Using NH_4Cl

Figure 29 shows the growth curves of nitrogen depleted cultures using NH_4Cl as sole nitrogen source. These curves are similar in shape to those obtained with B. stearothermophilus NCIB 8919 (Figure 24).

The plot of E_{420} maximum values against the initial NH_4Cl concentrations used is shown in Figure 28. The maximum optical density obtained under the experimental condition was slightly lower with nitrogen depleted cultures than with glucose

FIGURE 27

GROWTH CURVES OF GLUCOSE DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

All concentrations in molar

●—● 2.50×10^{-4} M.

▼—▼ 1.25×10^{-3} M.

◇—◇ 3.50×10^{-3} M.

◆—◆ 5.00×10^{-3} M.

□—□ 8.00×10^{-3} M.

△—△ 1.20×10^{-2} M.

Note: Time scale displaced 1 hr. for each curve.

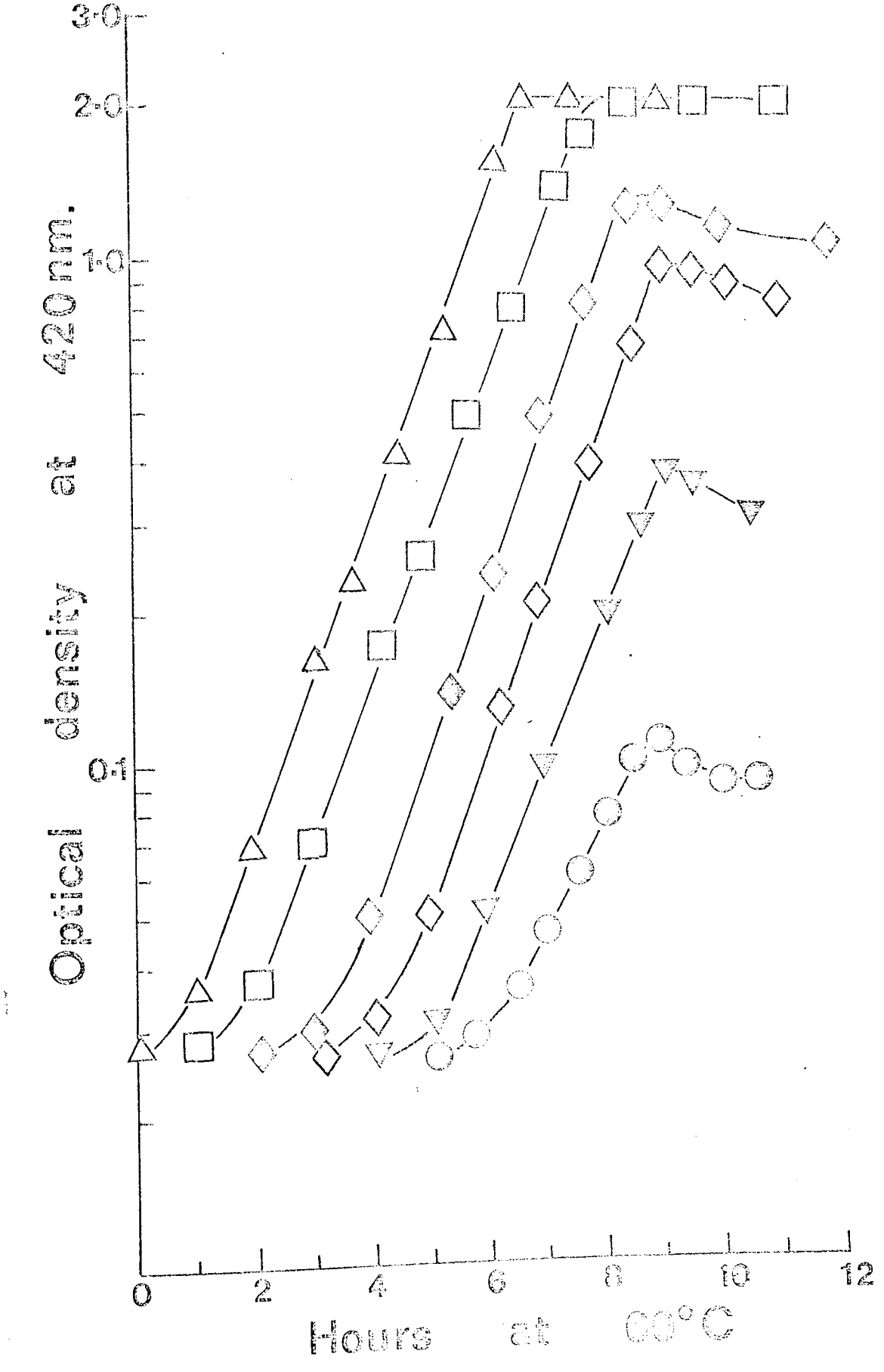

3·0
2·0
1·0
0·1
Optical density at 420 nm.
0
2
4
6
8
10
12
Hours at 60°C

FIGURE 28

THE RELATIONSHIP BETWEEN E_{420} (MAXIMUM) AND DIFFERENT INITIAL CONCENTRATIONS OF GLUCOSE OR NITROGEN USED IN THE CULTIVATION OF *B. STEAROTHERMOPHILUS* NCTC 10,003 (MUTANT)

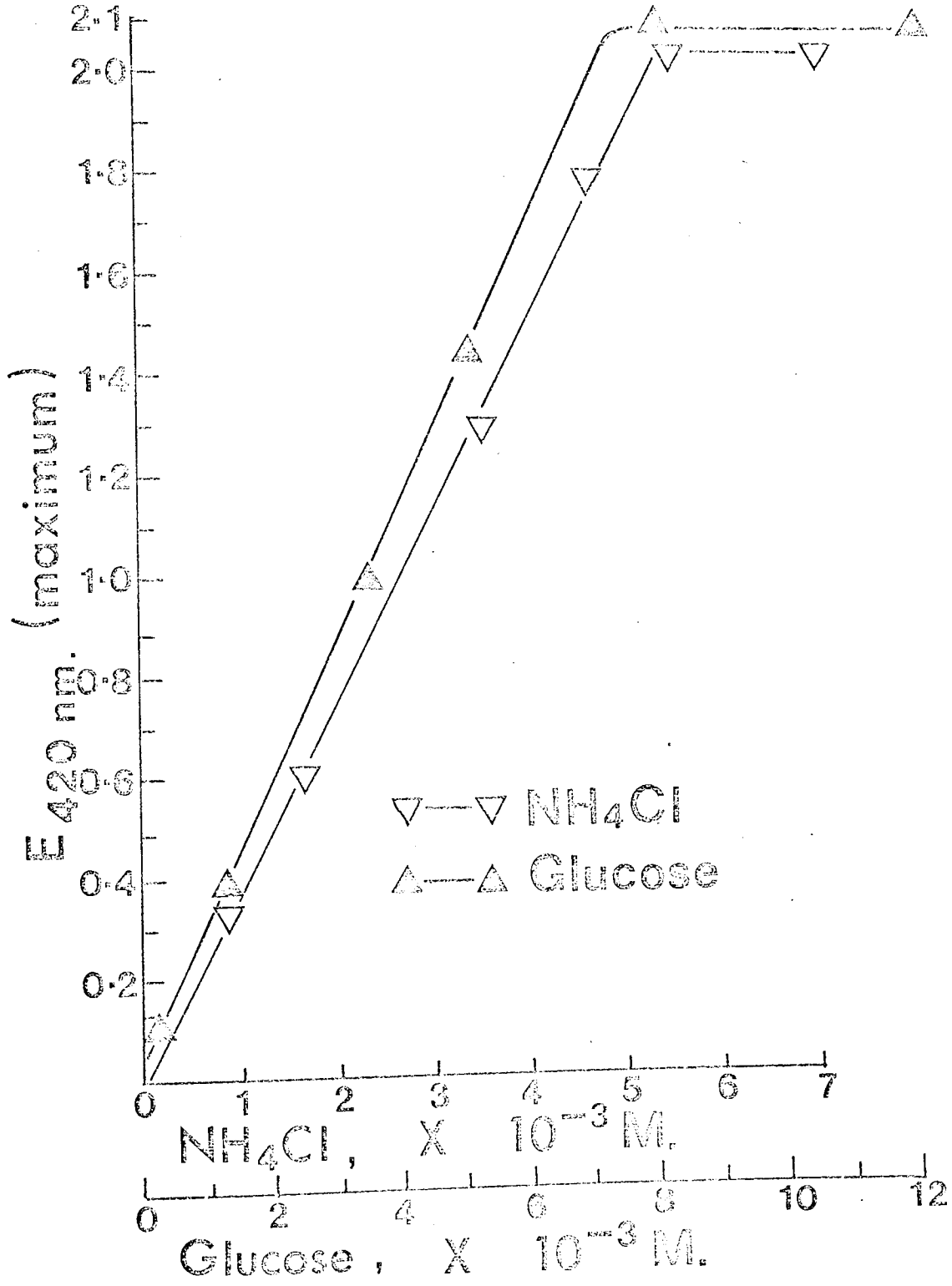

E 420 nm. (maximum)
2·1
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
▽—▽ NH4Cl
△—△ Glucose
0 1 2 3 4 5 6 7
NH4Cl, X 10⁻³ M.
0 2 4 6 8 10 12
Glucose, X 10⁻³ M.

FIGURE 29

GROWTH CURVES OF NITROGEN DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

All concentrations in molar.

■—■ 8.40×10^{-4} M.

◆—◆ 1.59×10^{-3} M.

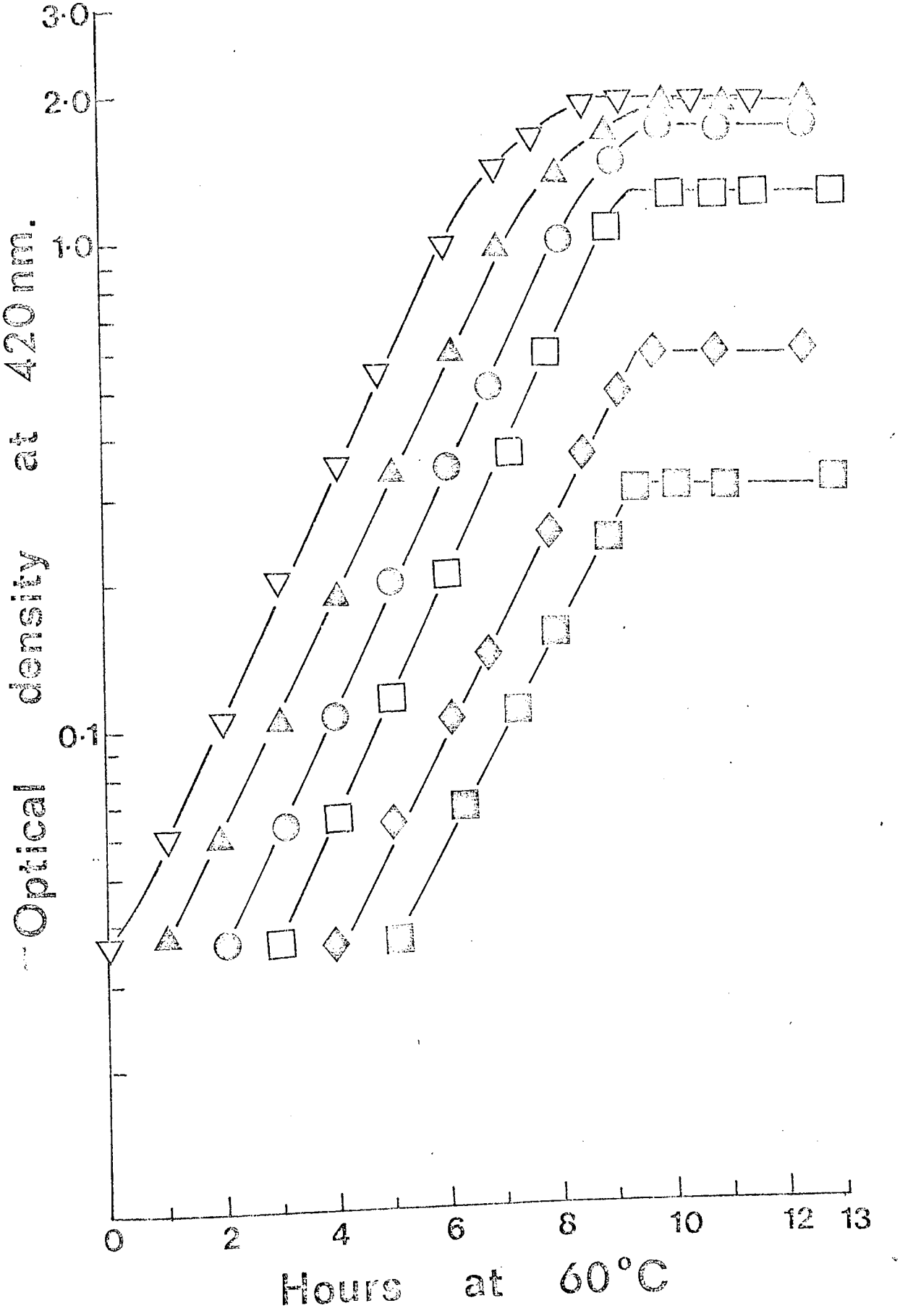
□—□ 3.55×10^{-3} M.

●—● 4.64×10^{-3} M.

▲—▲ 5.50×10^{-3} M.

▽—▽ 6.50×10^{-3} M.

Note: Time scale displaced 1 hr. for each curve.


3·0
2·0
1·0
0·1
Optical density at 420 nm.
0
2
4
6
8
10
12
13
Hours at 60°C

depleted cultures. This slight difference may be attributed to the difference in optical densities of the cells under the two culturing conditions.

(d) Glutamic Acid As A Carbon Source

Figure 30 shows the growth curves obtained with cultures containing a fixed concentration of glucose (1.0×10^{-3} M.) plus graded concentrations of glutamic acid.

In the presence of two carbon sources, growth was biphasic. Growth ceased temporary at an optical density reading corresponding to the depletion of glucose, suggesting that glucose was used first in preference to glutamic acid. The fact that cultures with progressively higher glutamic acid concentrations showed progressively higher first phases of growth indicated that the glutamic acid used was contaminated with utilizable glucose-like carbon compound. The growth rate of bacteria in glutamic acid was very much slower than in glucose. The doubling time in glutamic acid and glucose was about 240 minutes and 54 minutes respectively.

When E_{420} maximum values obtained from the growth curves were plotted against the concentrations of glutamic acid used, a linear relationship was obtained (Figure 31). The straight line intercepts on the Y-axis corresponded to the optical density provided by the fixed concentration of glucose.

FIGURE 30

GROWTH CURVES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) IN DEFINED MEDIA CONTAINING A FIXED CONCENTRATION OF GLUCOSE (1.0×10^{-3} M.) AND VARYING CONCENTRATIONS OF GLUTAMIC ACID

Glutamic acid concentration in molar.

◆—◆ Blank

▲—▲ 1.2×10^{-3} M.

○—○ 1.8×10^{-3} M.

■—■ 2.4×10^{-3} M.

◇—◇ 3.6×10^{-3} M.

Note: Time scale displaced 1 hr. for each curve.

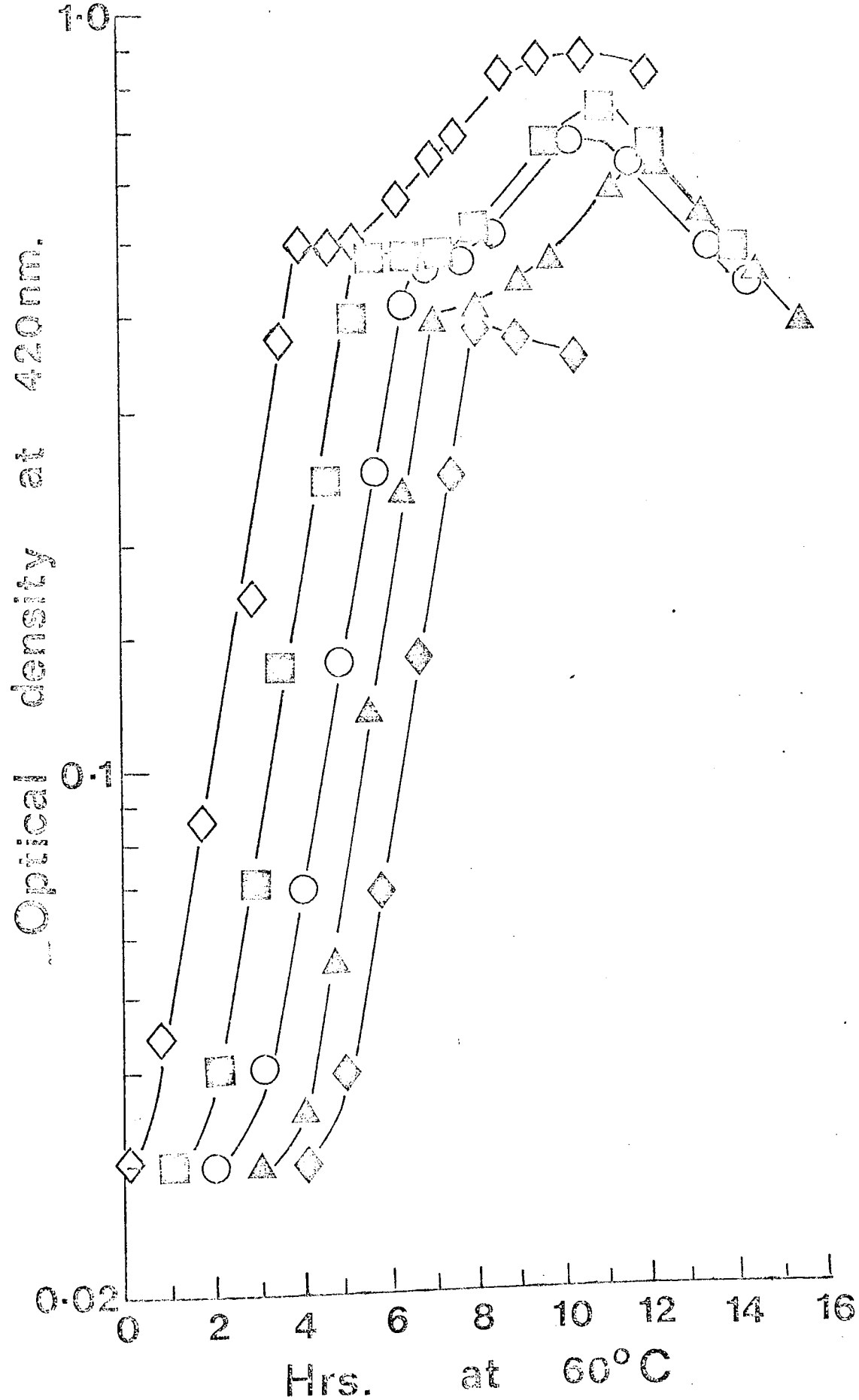
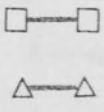

1·0
0·1
0·02
Optical density at 420 nm.
0
2
4
6
8
10
12
14
16
Hrs. at 60°C

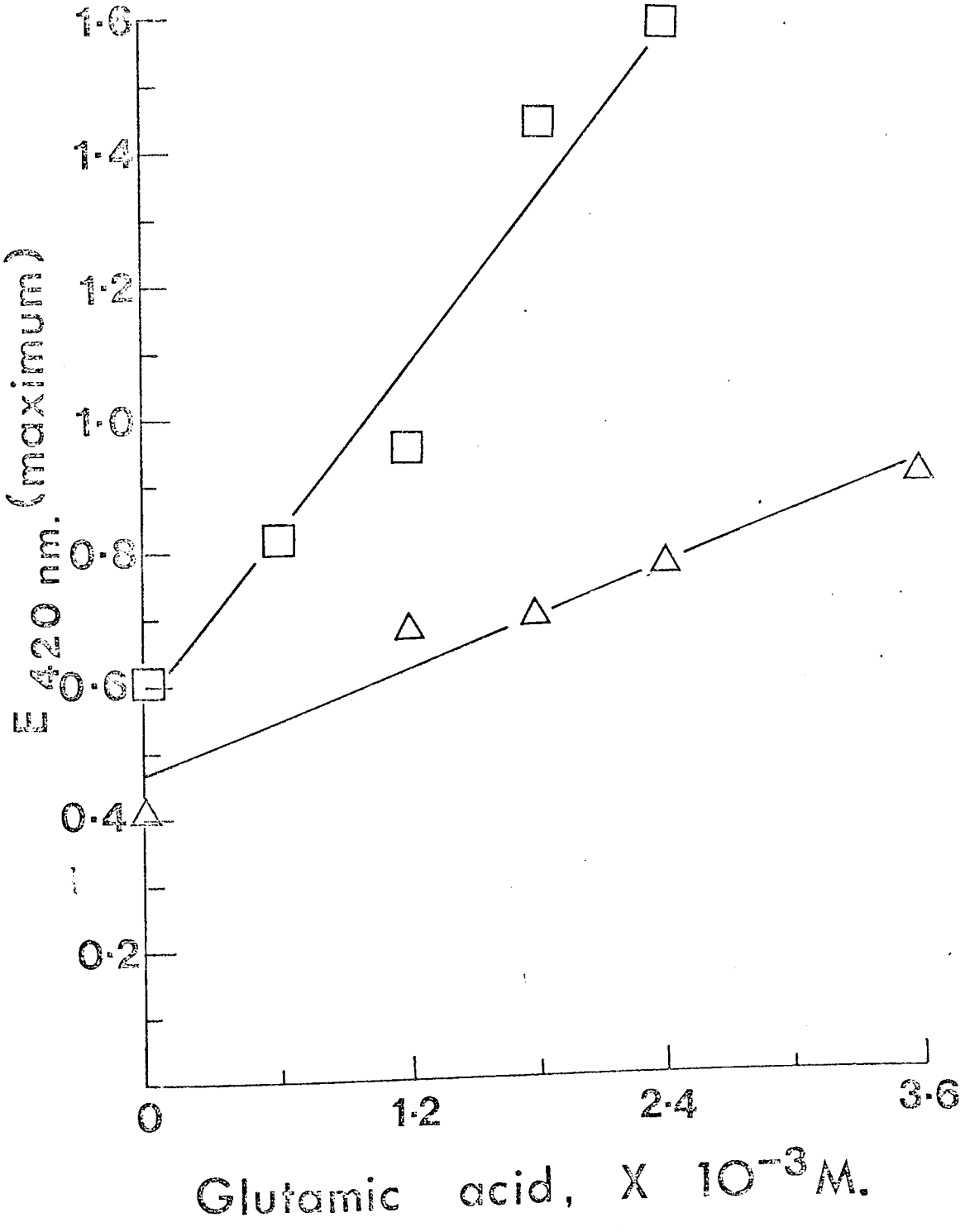
FIGURE 31

THE RELATIONSHIP BETWEEN E_{420} (MAXIMUM) AND DIFFERENT INITIAL CONCENTRATIONS OF GLUTAMIC ACID USED AS A CARBON OR A NITROGEN SOURCE



□—□ As a nitrogen source

△—△ As a carbon source


1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
E 420 nm. (maximum)
0
1·2
2·4
3·6
Glutamic acid, X 10^{-3} M.

(e) Glutamic Acid As A Nitrogen Source

Figure 32 shows the growth curves obtained with cultures containing a fixed concentration of NH_4Cl (1.87×10^{-3} M.) plus graded concentrations of glutamic acid.

Again, growth was biphasic in the presence of two nitrogen sources, with NH_4^+ being used in preference to glutamic acid. Cultures with progressively higher concentrations of glutamic acid also showed progressively higher first phases of growth, showing the contamination of glutamic acid with utilizable NH_4^+ - like compounds.

The plot of E_{420} maximum values obtained from the growth curves against the concentrations of glutamic acid used are shown in Figure 31. The straight line intercepts on the Y-axis corresponded to the optical optical density provided by the fixed concentration of NH_4Cl used.

(f) Sulphate And Magnesium Depletions

Figures 33 and 34 show the growth curves of sulphate and magnesium depleted cultures respectively. The shape of the curves are similar in shape to those obtained with Bacillus stearothermophilus NCIB 8919 during methionine and magnesium depletion. (Figures 19 and 26 respectively).

The relationship between E_{420} maximum values and the concentrations of sulphate or magnesium used are shown in

FIGURE 32

GROWTH CURVES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) IN DEFINED MEDIA CONTAINING A FIXED CONCENTRATION OF NH_4Cl (1.87×10^{-3} M.) AND VARYING CONCENTRATIONS OF GLUTAMIC ACID

Glutamic acid concentration in molar

◇—◇ Blank

○—○ 6.0×10^{-4} M.

●—● 1.2×10^{-3} M.

□—□ 1.8×10^{-3} M.

◆—◆ 2.4×10^{-3} M.

Note: Time scale displaced 1 hr. for each curve.

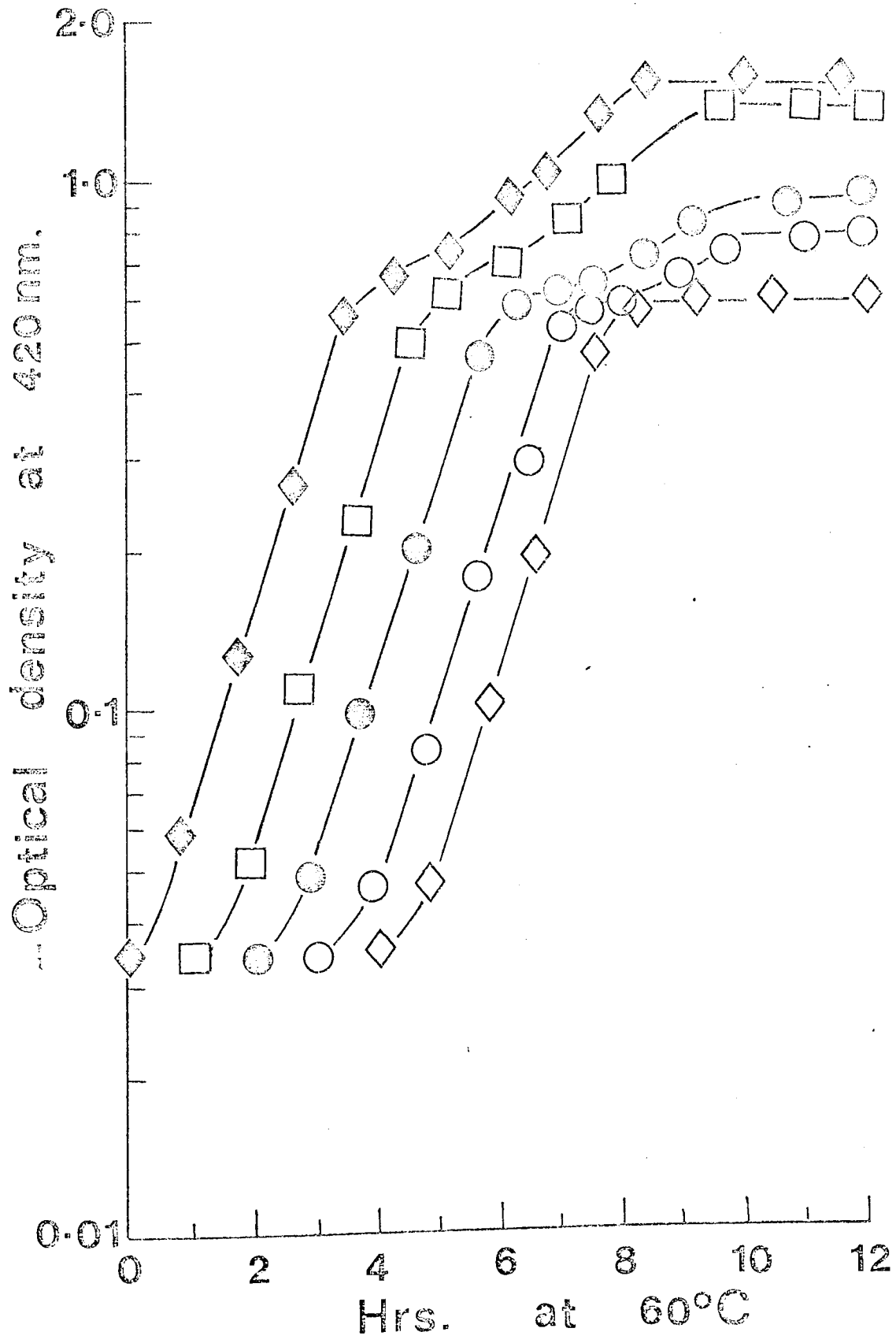

2·0
1·0
0·1
0·01
Optical density at 420 nm.
0
2
4
6
8
10
12
Hrs. at 60°C

FIGURE 33

GROWTH CURVES OF SULPHATE DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

All concentrations in molar.

●—● 7.5×10^{-6} M.

■—■ 2.5×10^{-5} M.

◆—◆ 4.5×10^{-5} M.

◇—◇ 7.0×10^{-5} M.

▲—▲ 9.5×10^{-5} M.

△—△ 1.2×10^{-4} M.

Note: Time scale displaced 1 hr. for each curve.

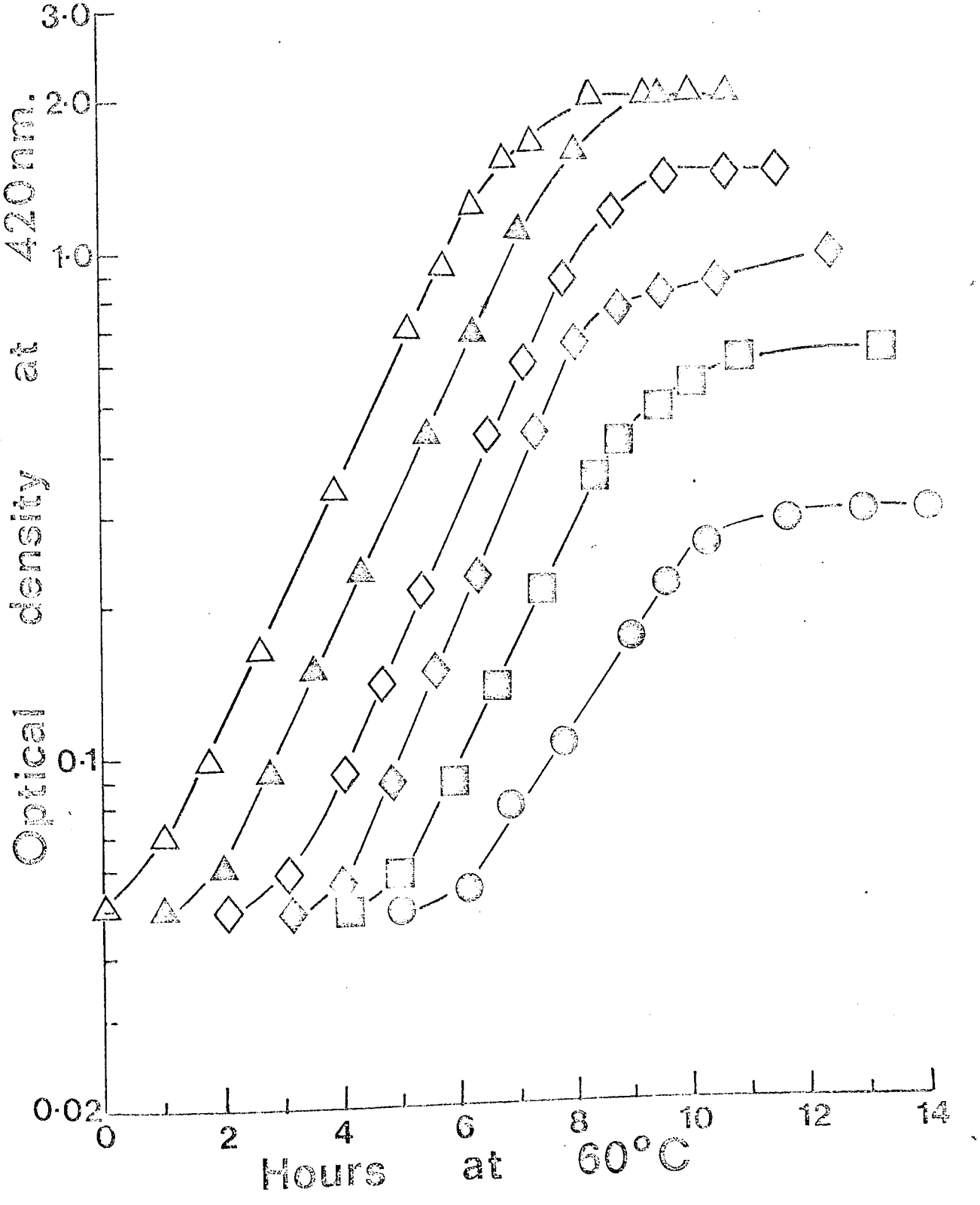

Optical density at 420 nm.
3·0
2·0
1·0
0·1
0·02
0
2
4
6
8
10
12
14
Hours at 60° C

FIGURE 34

GROWTH CURVES OF MAGNESIUM DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

All concentrations in molar.

□—□ 2.60×10^{-6} M.

●—● 1.30×10^{-5} M.

△—△ 1.75×10^{-5} M.

◆—◆ 2.34×10^{-5} M.

▼—▼ 3.38×10^{-5} M.

◇—◇ 5.00×10^{-5} M.

Note: Time scale displaced 1 hr. for each curve.

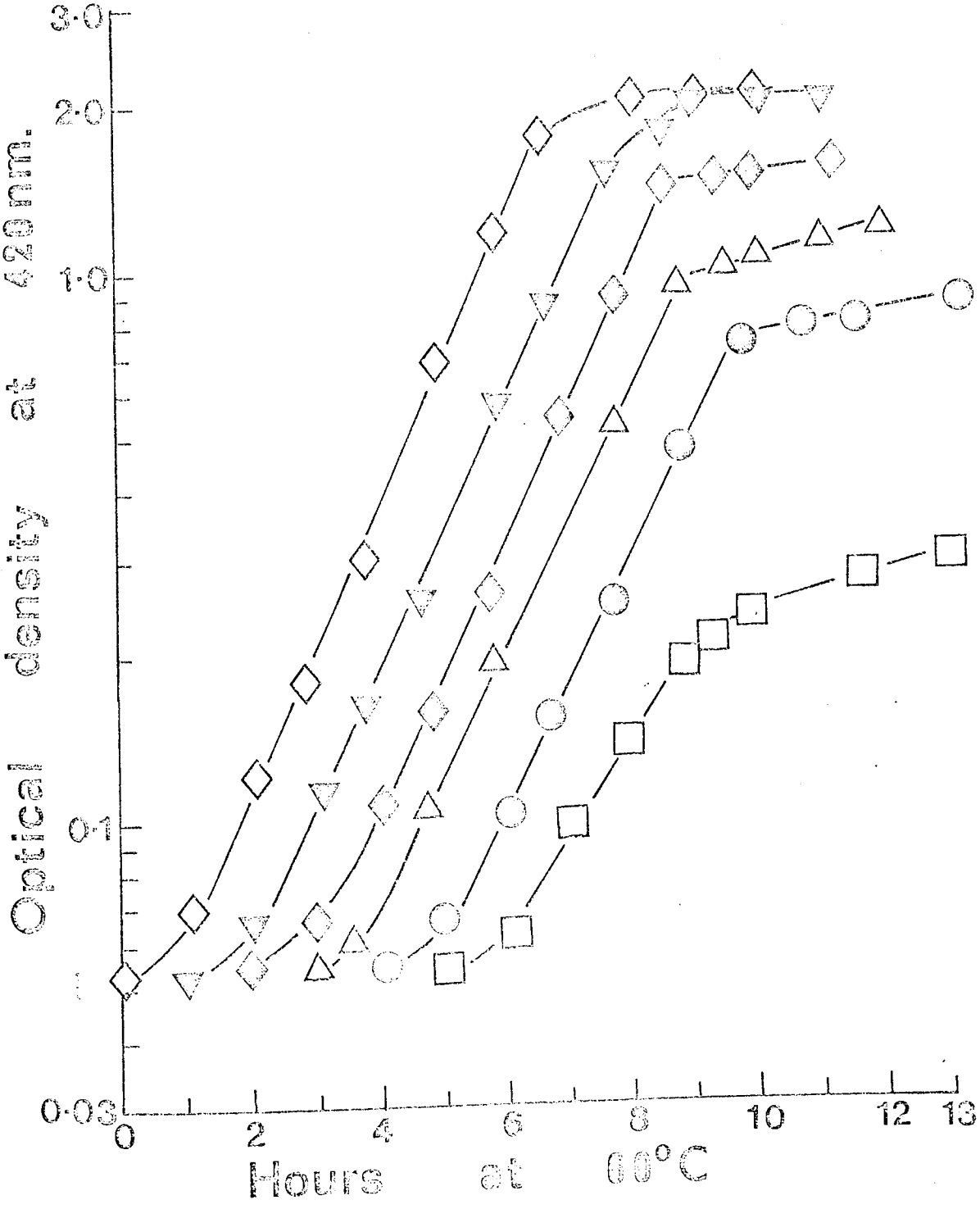

Optical density at 420 nm.
3·0
2·0
1·0
0·1
0·03
0
2
4
6
8
10
12
13
Hours at 60°C

FIGURE 35

THE RELATIONSHIP BETWEEN E_{420} (AFTER 8 HOURS) AND DIFFERENT INITIAL CONCENTRATIONS OF MAGNESIUM OR SULPHATE USED IN THE CULTIVATION OF *B. STEAROTHERMOPHILUS* NCTC 10,003 (MUTANT)

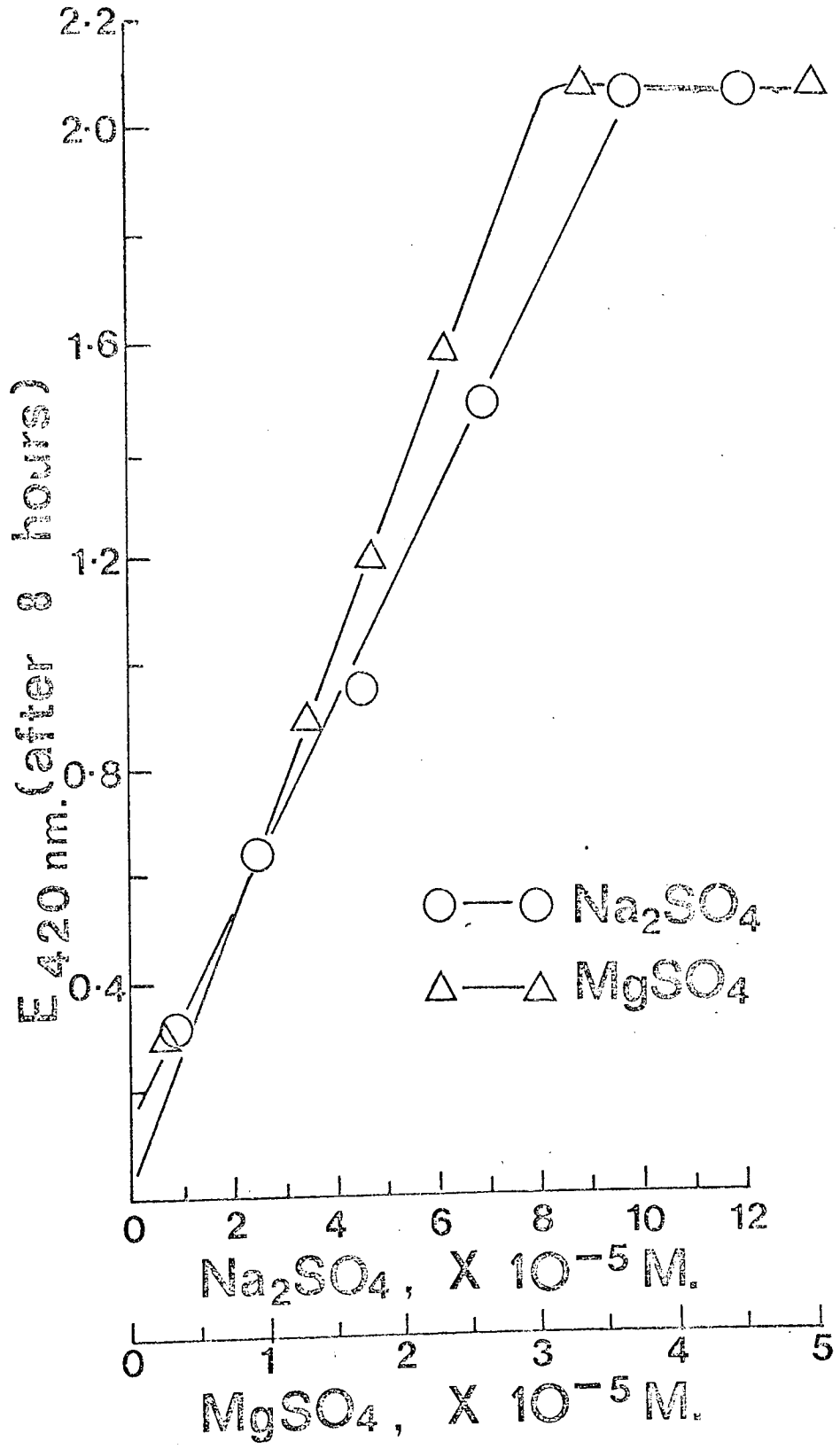

2·2
2·0
1·6
1·2
0·8
0·4
E 420 nm. (after 8 hours)
O—O Na2SO4
Δ—Δ MgSO4
0
2
4
6
8
10
12
Na2SO4, X 10−5 M.
0
1
2
3
4
5
MgSO4, X 10−5 M.

Figure 35. The fact that both straight lines did not pass through the origin indicated that the medium contained some contaminating levels of ions. The level of contamination was relatively high with sulphate (about 8×10^{-4} M.) and that of magnesium was about 2×10^{-6} M.

(g) Phosphate Depletion

Figure 36 shows the growth curves of phosphate depleted cultures. The relationship between E_{420} and the initial concentrations of phosphate used are shown in Figure 37. A linear relationship was obtained up to E_{420} of 0.8.

Since all other cultures gave maximum growth of E_{420} 2.1 to 2.2, it is conceivable that the factors that limited further growth in phosphate depleted cultures were different from that of other cultures studied. It is likely that the replacement of phosphate buffer, a major component in the defined medium, with HEPES buffer could result in the removal of some trace elements present in the phosphate. The trace elements might well be critical for growth. The effect of trace elements required for growth was not investigated in the present study.

The straight line depicted in Figure 37 intercepts the Y-axis, corresponded to a contaminating level of 5×10^{-6} M. phosphate.

(h) Iron, Calcium And Manganese Depletions

Cultures depleted of iron, calcium or manganese showed no

FIGURE 36

GROWTH CURVES OF PHOSPHATE DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

All concentrations in molar.

●—● 5.0×10^{-6} M.

□—□ 3.0×10^{-5} M.

▲—▲ 5.0×10^{-5} M.

◆—◆ 6.0×10^{-5} M.

○—○ 8.0×10^{-5} M.

△—△ 1.0×10^{-4} M.

Note: Time scale displaced 1 hr. for each curve.

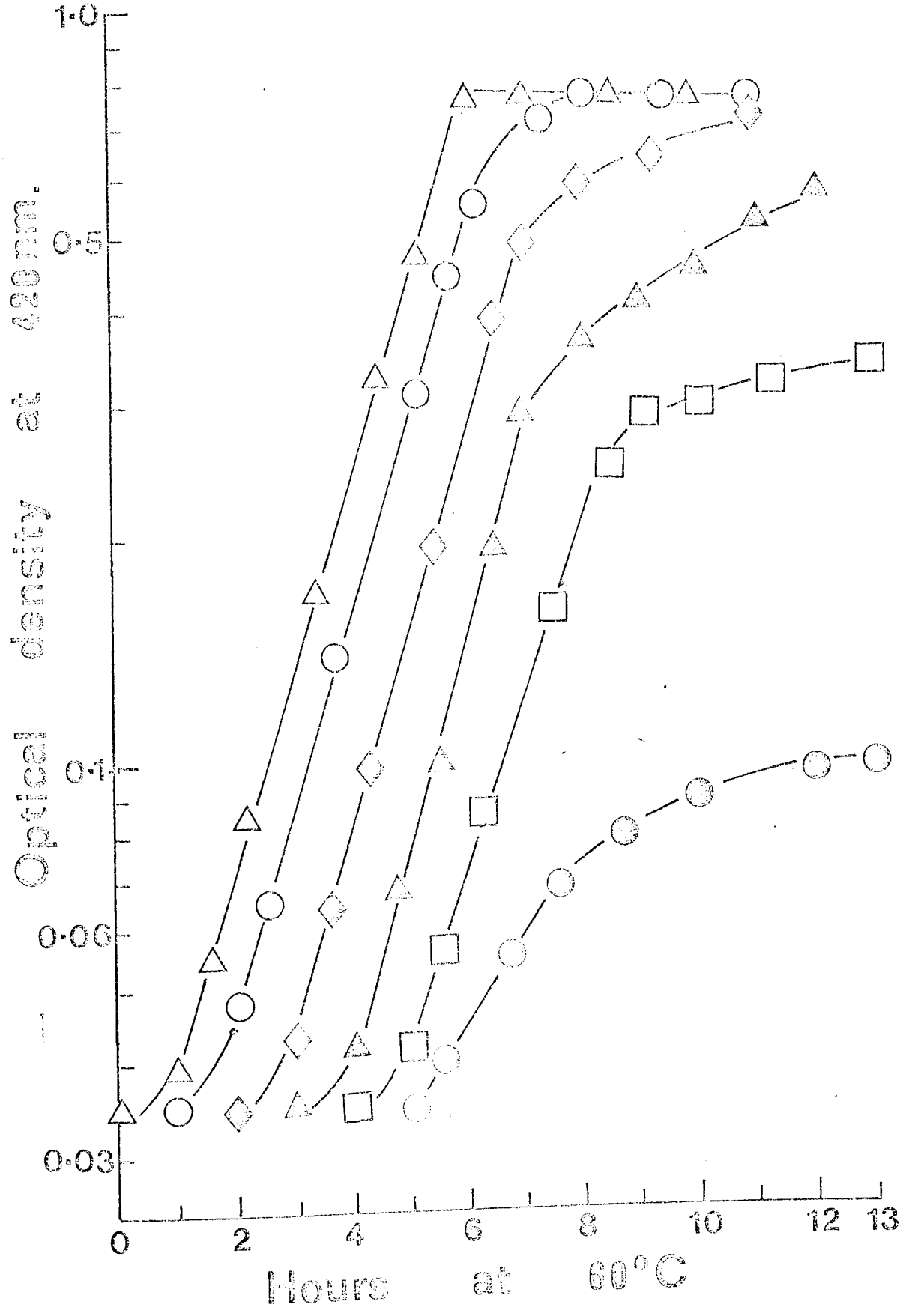
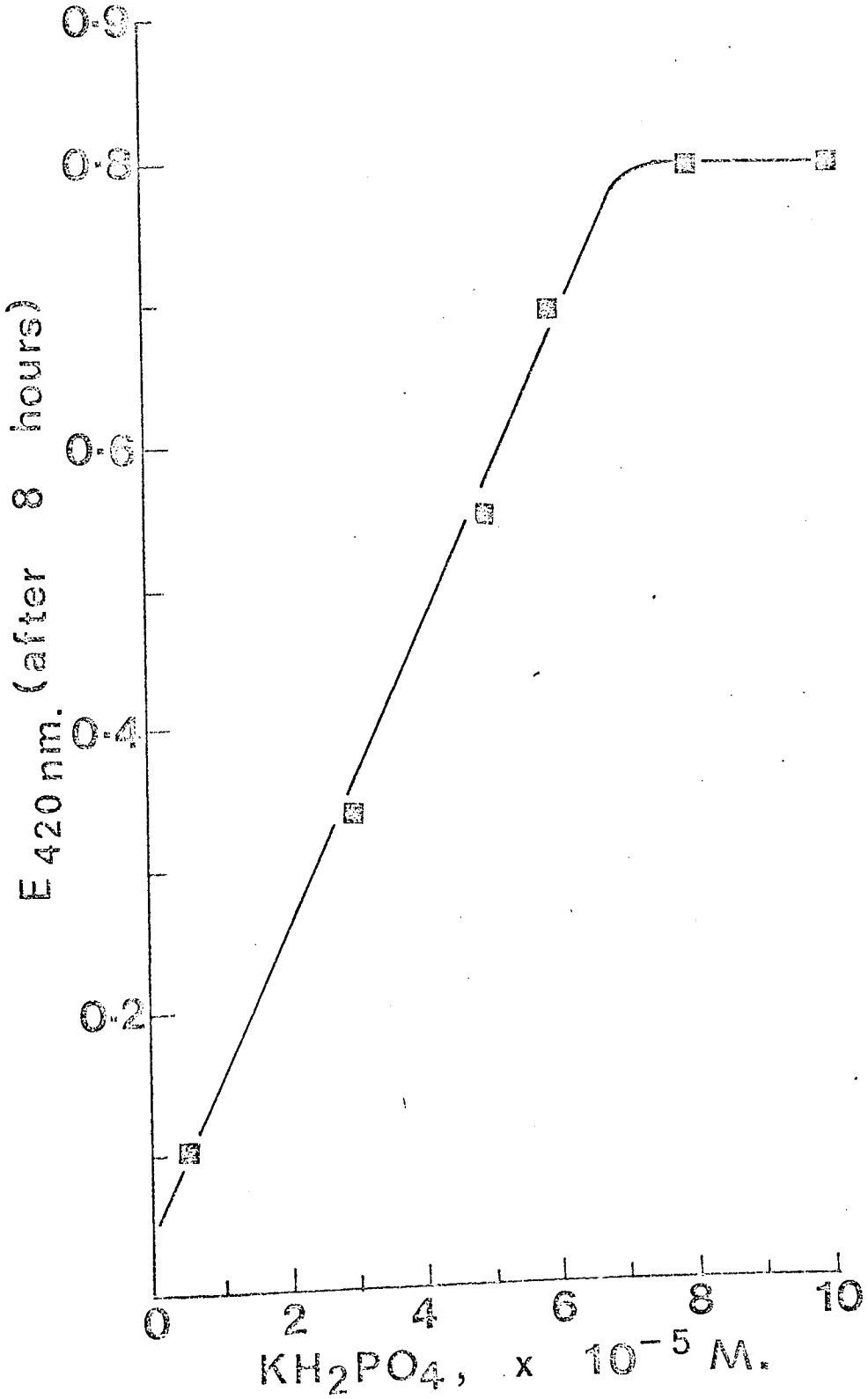

1·0
0·5
0·1
0·06
0·03
Optical density at 420 nm.
0
2
4
6
8
10
12
13
Hours at 60°C

FIGURE 37

THE RELATIONSHIP BETWEEN E_{420} (AFTER 8 HOURS) AND DIFFERENT INITIAL CONCENTRATIONS OF PHOSPHATE USED IN THE CULTIVATION OF *B. STEAROTHERMOPHILUS* NCTC 10,003 (MUTANT)


0·9
0·8
0·6
0·4
0·2
E 420 nm. (after 8 hours)
0
2
4
6
8
10
KH2PO4, x 10−5 M.

reduction in maximum growth. It was concluded that the requirements for calcium, iron or manganese were low and were satisfied by the level of impurities present in other medium components.

(i) Microscopic Examination Of Cultures Under Different Depleting Conditions

With the exception of magnesium and manganese cultures, all other cultures studied formed spores. Sporulation was low in the absence of added glutamic acid. The spores formed under various nutrient depleted conditions appeared similiar in phase-contrast microscopy.

5. FACTORS AFFECTING THE DEGREE OF SPORULATION

A. INTRODUCTION

B. stearothermophilus NCTC 10,003 (mutant) was chosen for further studies because it is less fastidious in its nutritional requirements than other strains studied. Moreover, preliminary studies had indicated a greater spore yield with this strain in the presence of added glutamic acid.

Sporulation in aerobic sporeformers is determined to a great extent by the oxygen availability of the culture. The oxygen availability per cell in a liquid culture is undoubtedly related to the cell density.

In the present study, whenever possible, the cell density of the culture was kept constant by making use of data obtained from growth depletion studies described in Section 4.B.

B. EXPERIMENTAL AND RESULTS

(a) The Effect Of Specific Nutrient Depletion

The composition of media used to determine the effect of specific nutrient depletions on percentage sporulation are shown in Table 17. The concentrations of $MgSO_4$, $FeSO_4$, $MnSO_4$ and $CaCl_2$ used were adopted from medium previously used by Anderson and Friesen (1972). NH_4Cl was used at a concentration which, together with nitrogen from glutamic acid, would theoretically support growth to E_{420} of 2.5.

TABLE 17

THE EFFECT OF SPECIFIC NUTRIENT DEPLETED GROWTH ON PERCENTAGE SPORULATION OF B.STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

Depletion → / Medium component ↓	Carbon	Nitrogen	Magnesium and Carbon	Sulphate	Phosphate
Na_2HPO_4	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}	
KH_2PO_4	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}	7.30×10^{-3}	6.50×10^{-5}
NH_4Cl	4.00×10^{-3}		4.00×10^{-3}	4.00×10^{-3}	4.00×10^{-3}
$MgSO_4.7H_2O$	5.00×10^{-4}	5.00×10^{-4}	1.40×10^{-5}		5.00×10^{-4}
$FeSO_4.7H_2O$	1.00×10^{-5}	1.00×10^{-5}	1.00×10^{-5}		1.00×10^{-5}
$MnSO_4.7H_2O$	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}		1.00×10^{-4}
$CaCl_2.6H_2O$	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}
Glucose	1.50×10^{-3}	7.50×10^{-3}	1.50×10^{-3}	7.50×10^{-3}	7.50×10^{-3}
L-Glutamic acid	2.40×10^{-3}	2.40×10^{-3}	2.40×10^{-3}	2.40×10^{-3}	2.40×10^{-3}
Na_2SO_4			4.86×10^{-4}	4.10×10^{-5}	
$MgCl_2.6H_2O$				5.00×10^{-5}	
$FeCl_2.4H_2O$				1.00×10^{-5}	
$MnCl_2.4H_2O$				1.00×10^{-4}	
HEPES					3.00×10^{-2}
KCl					7.23×10^{-3}
NaCl					1.76×10^{-2}
Percentage sporulation	35.8	24.8	12.9	72.2	34.8

Magnesium depleted culture did not sporulate but culture depleted of magnesium and carbon simultaneously did. The percentage of sporulation in culture depleted of magnesium-carbon simultaneously was determined.

Growth was carried out with 25 ml. cultures contained in 100 ml. conical flasks using a Mickle reciprocal shaker as described in Section 2.R. The percentage of sporulation was assessed after incubation at 60°C for 15 hours. The method used for the determination of percentage sporulation has been described in Section 2.H.

With the exception of culture in which phosphate was depleting (PO_4-), all other cultures were growth depleted at optical density of 1.0 to 1.1. The PO_4- culture was growth depleted at optical density of 0.70 to 0.75 because the maximum optical density possible was 0.8 (Figure 37).

The percentage of sporulation with different nutrient depleted cultures are shown at the bottom of Table 17. It can be seen that sulphate depleted growth (SO_4-) gave the highest percentage of sporulation (72.2%). Sporulation was low when the culture was depleted simultaneously of magnesium and carbon (Mg-C-), 12.9%. All other nutrient depleted cultures showed intermediate degree of sporulation (25-36%).

(b) The Effect Of Aeration Rate

The effect of culture aeration on percentage sporulation

was studied by batch cultivation using the chemostat vessel as described in Section 2.F. This was necessary because this instrument permitted the rigid control of air-sparging rate as well as the stirring rate of the culture, which would otherwise has been impossible to perform in a Mickle reciprocal shaker. Moreover, it was essential to determine the optimal operating conditions of the chemostat which was later batch-used for large scale production of spores. Cultures were carbon depleted at E_{420} of 1.0 to 1.1 (Table 17, column 1) and the effect of sparging rate cum stirring rate on percentage sporulation was determined. The method used for the determination of percentage sporulation has been described in Section 2.H. and the results are shown in Table 18. It can be seen that percentage sporulation was low (6.5%) at low stirring rate. This may be attributed to the low efficiency of oxygen absorption due to large air bubble size and decreased gas-liquid interface at low stirring rates. At higher rates, smaller bubbles and increased interfacial area improved absorption.

The aeration of the culture was invariably determined by the stirring and sparging rates. Optimal sporulation (35.8%) occurred at a sparging rate of 1200 ml. air/minute/litre culture and a stirring rate of 400 r.p.m. Since the unsparged, shaker culture using a reciprocal shaker operating at 110 x 3.8 cm. throws per minute gave similar high degree of sporulation as the optimally sparged/stirred culture. This showed that the shaking culture was optimally aerated at the quoted throw speed.

TABLE 18

THE EFFECT OF AERATION RATE ON PERCENTAGE SPORULATION OF CARBON DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

SPARGING RATE (ml. air/mn./L culture)	STIRRING RATE (r.p.m.)	% SPORULATION
714	100	6.5
714	300	34.3
1200	400	35.8
1657	500	34.8
Unsparged shaking culture operating at 110 x 3.8 cm. throws/minute		35.7

(c) The Effect Of Manganese And Calcium Concentration

This was studied with 25 ml. cultures using Mickle reciprocal shaker operating at the optimal aeration level (110 x 3.8 cm. throws per minute). The method used has been described in Section 2.R. Cultures were carbon depleted at E_{420} of 1.0 to 1.1 and the percentage of spores formed determined after 15 hours of incubation.

The results are presented in Tables 19 and 20. It can be seen that the manganese concentration of the medium affects the percentage of sporulation, with 1.0×10^{-4} M. manganese appearing to be the optimal level. The sporulation percentage was not appreciably affected by the concentration of calcium though 1.0×10^{-4} M. calcium appeared to give a slightly higher percentage of sporulation. This confirmed the observation of Slepecky and Foster (1959) that manganese is required for spore production on synthetic medium.

(d) The Effect Of Glucose And Glutamic Acid Concentration

The effect of various combinations of glucose and glutamic acid in the medium on percentage sporulation is shown in Table 21.

Experiments were carried out using 25 ml. cultures in a Mickle reciprocal shaker as described in Section 2.R. The maximum optical density of the culture varied from E_{420} of 0.45 to 2.1 thus, the oxygen availability per cell varied from culture to culture. In the present study, the culture was agitated

TABLE 19

THE EFFECT OF MANGANESE CONCENTRATION ON PERCENTAGE SPORULATION OF CARBON DEPLETED CULTURES OF *B. STEAROTHERMOPHILUS* NCTC 10,003 (MUTANT)

MANGANESE CONCENTRATION (IN MOLAR)	% SPORULATION
0	3.6
1.0×10^{-6}	13.9
5.0×10^{-6}	18.5
1.0×10^{-5}	19.0
1.0×10^{-4}	38.8
2.0×10^{-4}	35.6
3.0×10^{-4}	Precipitation

TABLE 20

THE EFFECT OF CALCIUM CONCENTRATION ON PERCENTAGE SPORULATION OF CARBON DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

CALCIUM CONCENTRATION (IN MOLAR)	% SPORULATION
0	33.4
1.0×10^{-6}	34.5
5.0×10^{-6}	32.5
1.0×10^{-5}	34.8
1.0×10^{-4}	39.2
1.4×10^{-4}	36.5
2.0×10^{-4}	Precipitation

TABLE 21

PERCENTAGE SPORULATION OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) IN MEDIA CONTAINING VARIOUS AMOUNT OF GLUCOSE AND GLUTAMIC ACIDS

GLUCOSE (IN MOLAR)	GLUTAMIC ACID (IN MOLAR)				
	0	6.0×10^{-4}	1.2×10^{-3}	2.4×10^{-3}	4.8×10^{-3}
2.0×10^{-3}	5.2	14.3	31.2	35.7	32.0
3.0×10^{-3}	7.3	9.1	24.2	32.0	31.5
3.5×10^{-3}				28.2	
4.5×10^{-3}				31.8	
6.0×10^{-3}				30.5	

at 110 x 3.8 cm. throws per minute. This corresponds to the optimum aeration level for culture growth depleted at E_{420} of 1.0 (Table 18). The results of Table 18 also showed that culture aerated well above the optimum level (1200 ml. of air/minute/litre culture and stirring rate of 400 r.p.m.) sporulated optimally. Thus, the higher rate of aeration as occurred in culture with low optical density reading, was without effect on percentage sporulation and the data obtained were still useful.

The optimal combination appeared to be 2.0×10^{-3} M. glucose and 2.4×10^{-3} M. glutamic acid. Sporulation decreased slightly with increased in the concentration of either glucose or glutamic acid. This was presumably due to higher population density, resulting in the oxygen availability per cell being reduced.

6. SPORE PROPERTIES AND COMPOSITION OF THE SPORULATION MEDIA

The main objective of the present study was to design media, chemically defined and suitable for the production of Bacillus stearothermophilus spores. It was envisaged that the spores prepared would be of consistent composition, dormancy and resistance. An applied objective was that such spores could then be used as biological indicator in sterilization processes.

It is well known that the chemical composition of the growth medium determined the chemical composition of the spores produced. Changes in the chemical composition of the spores have inturn been correlated with changes in spore properties. In this section of the work, some effect of changes in the medium composition on spore properties were examined. The properties that were studied included the chemistry (dipicolinic acid, diaminopimelic acid, hexosamine and cation content), heat inactivation and germination of spores. The method employed have been described in Section 2.I. to 2.O.

A. THE EFFECT OF SPECIFIC NUTRIENT DEPLETION ON THE PROPERTIES OF BACILLUS STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES

Percentage sporulation studies have indicated that specific nutrient depletion greatly affected the extent of sporulation. It was thus logical that other effects of specific nutrient depletion should also be studied.

Table 17 shows the composition of media used to prepare spores

of various kinds each depleted of one or two specific nutrients. Spores were prepared by batch cultivation using a chemostat vessel as described in Section 2.F. The harvesting and cleaning of spores have been described in Section 2.G. The spores used for detailed studies were almost totally phase-bright and free of clumps after suspension in 0.05 M. saline. The percentage of phase-dark spores and non-spore objects if present was less than 3%. Samples with contaminants higher than this level were not used and discarded.

The heat inactivation curves of the various spore types were determined as described in Section 2.N. and the results are shown in Figure 38. With the exception of spores from sulphate depleted culture (SO_4-), heating produced a rapid initial drop in viability followed by a less abrupt exponential decline. The extent of initial drop in viability depended on the nature of nutrient depletion, being greater for spores from carbon depleted cultures (C-) and phosphate depleted cultures (PO_4-) and less so with spores from nitrogen depleted cultures (N-) and cultures depleted of magnesium and carbon simultaneously (Mg-C-). The slope of the exponential decline was also determined by the nature of growth depletion, being steepest with C- spores and least so with N- spores. SO_4- spores exhibited three phases of response on heating, a rapid initial increase in colony count in the initial 5 minutes of heating, followed by a rapid decline in colony count and finally the exponential decline phase similar to those exhibited by other spore types.

FIGURE 38

HEAT INACTIVATION OF SPORES FROM SPECIFIC NUTRIENT DEPLETED CULTURES OF *B. STEAROTHERMOPHILUS* NCTC 10,003 (MUTANT)

Spores obtained from cultures depleted of:

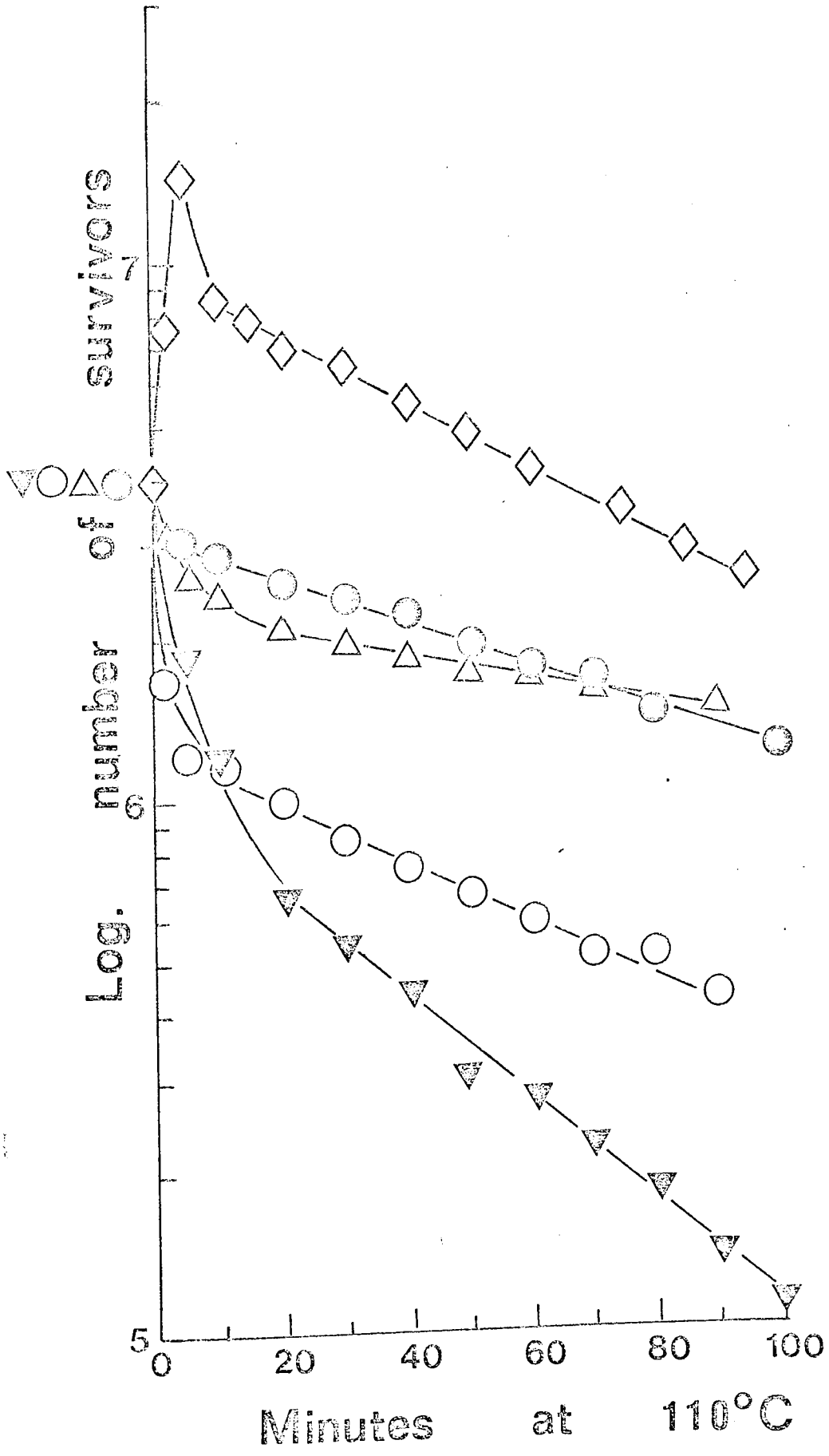
◇—◇ Sulphate

△—△ Nitrogen

●—● Magnesium and carbon simultaneously

○—○ Phosphate

▽—▽ Carbon alone


Log. number of survivors
7
6
5
0
20
40
60
80
100
Minutes at 110°C

Survival curves of biphasic responses (sometimes described as concave upward) have been attributed to the presence of populations of two discrete levels of resistance (Plug and Schmidt, 1968). Consequently, the Y-intercept of the second component of survival curve would represent the proportion of discrete resistant population at time zero.

In the present study, some preliminary experiments had shown that the survival curves of spores grown from survivors of spores heated at 110°C for 100 minutes were similar to those of the original spore stock, indicating that the initial rapid decline and subsequent less steep decline was not due to two genetically different spore populations with discrete resistance levels. The spores may be phenotypically different due to the constant change of the chemical makeup of the sporulation medium during batch cultivation. Similarly shaped survival curves have been postulated as being due to the transformation of sensitive to resistant spores during heating (Alderton et al, 1964; Komenushi et al, 1966).

Nevertheless, for the sake of easy description, the inactivation curves of various kinds obtained here were compared by accepting that there were two spore populations with different levels of resistance perhaps phenotypically caused by batch cultivation. "D value" or decimal reduction time, the time taken in minute to destroy 90% of the spore population was computed from the slope of the inactivation curve. D_1 was taken as the D value of the sensitive population, D_2 was taken as the D value of

the resistant population and D_3 was the time taken to destroy 90% of the initial spore population (i.e. from time zero). %(R) was taken as the percentage of heat resistant population. This was determined from the Y-intercept of the resistant population as described above.

The effect of specific nutrient depletion on spore germination is shown in Figure 39. PO_4- and N- spores germinated rapidly and extensively without prior heat activation. Germination was completed after 25-30 minutes incubation at 60°C. The germination of Mg-C-; C- and SO_4- spores was sluggish and less extensive. The % of germination after 30 minutes incubation was used as a basis for comparison of the various spore types.

Spores were also analyzed for dipicolinic acid, diaminopimelic acid, hexosamine, calcium, magnesium and manganese contents as described in Section 2.I. to 2.L. and the results are summarised in Table 22, together with the heat resistance data (D_1; D_2; D_3 and %(R)) and % germination after 30 minutes incubation.

The dipicolinic acid and calcium contents of N- and Mg-C- spores were higher than other spore types. These spores also possessed higher heat resistance as shown by their higher D_1, D_2, D_3 and %(R) values. The molar ratio of Ca/D.P.A of all spore types were close to unity. This confirms the report of Murrell (1967) and Murrell and Warth (1969), they provided evidence that showed calcium increased significantly with increase in the heat resistance of spores and the molar ratio of D.P.A. to calcium of

FIGURE 39

GERMINATION OF SPORES FROM SPECIFIC NUTRIENT DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

Spores obtained from cultures depleted of:

▽—▽ Phosphate

△—△ Nitrogen

○—○ Magnesium and carbon simultaneously

□—□ Carbon alone

◇—◇ Sulphate

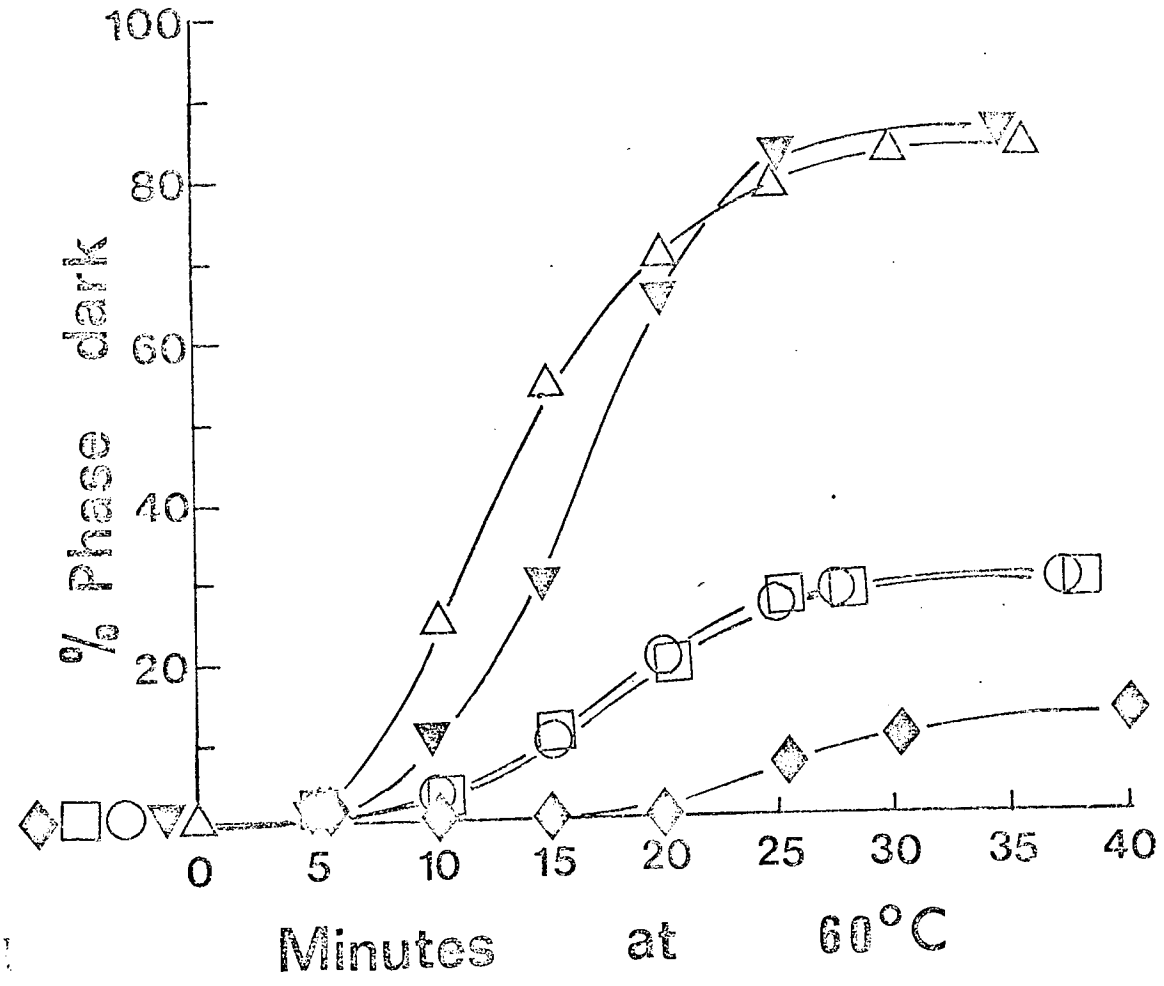

100
80
60
40
20
% Phase dark
0
5
10
15
20
25
30
35
40
Minutes at 60°C

TABLE 22

THE EFFECT OF SPECIFIC NUTRIENT DEPLETION ON THE PROPERTIES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES

	SPORE TYPE				
	N-	C-	Mg-C-	SO_4-	PO_4-
D.P.A. ($\mu g./10^7$ spores)	1.9560	1.6753	1.8518	1.3570	1.5050
Calcium ($x10^{-1} \mu g./10^7$ spores)	5.2000	3.7500	5.4070	3.9200	4.0000
$\frac{Ca}{D.P.A.}$ molar ratio	1.11	0.94	1.23	1.21	1.11
Magnesium ($x10^{-1} \mu g./10^7$ spores)	0.2500	0.7358	0.2560	0.3630	5.7170
$\frac{Mg}{Ca}$ molar ratio	0.013	0.3270	0.0790	0.1540	2.3800
Manganese ($x10^{-1} \mu g./10^7$ spores)	1.4090	1.2850	3.2410	1.2700	1.1000
Hexosamine ($\mu g./10^7$ spores)	1.1890	1.3470	0.6700	1.1800	2.0280
D.A.P. ($\mu g./10^7$ spores)	0.5384	0.5903	0.6716	0.4677	0.5360
$\frac{Hexosamine}{D.A.P.}$ molar ratio	2.42	2.50	1.09	2.77	4.15
% germination (after 30 mn. at 60°C	84	28	31	11	88
Heat inactivation at 110°C: D_1	~23	~11	~29		~5
D_2 in mn.	496	102	248	164	185
D_3	379	44	232	228	92
% (R)	55.6	25.8	78.3	66.6	31.6
* % dormant spores				87.0	

* % dormant spores = 100 - % viable spores

% viable spores calculated as: $\frac{\text{colony counts/ml.}}{\text{total counts/ml.}} \times 100$

most spores were close to one.

The magnesium content of Mg-C- and N- spores was low, this low concentration of magnesium appeared to be compensated by the corresponding increase in the calcium concentration. Murrell and Warth (1965) reported Mg/Ca molar ratios of 0.05 to 0.3. With the exception of PO_4- spores, the values obtained with all other spore types were close to this range. The Mg/Ca molar ratio was lower with Mg-C-, N- and SO_4- spores than PO_4- and C- spores. The N- and Mg-C- spores were more heat resistant than C- and PO_4- spores. SO_4- spores were slightly more resistant than C- spores with respect to D_2 value. The high %(R) value of SO_4- spores indicated that the majority of the spore population (66.6%) were of resistant type. The evidence seems to agree with the finding of Murrell and Warth (1965), they showed that Mg/Ca molar ratio decreased significantly with decrease in heat resistance ($P < 0.001$).

SO_4- spores were relatively low in D.P.A. compared with other spore types. These spores were also highly dormant as determined from the total and colony counts. This evidence however, is not so conclusive as to correlate the content of D.P.A. with spore dormancy.

PO_4- spores were typically rich in magnesium. The reason for this is not known. These spores were prepared in medium with HEPES as buffer as compared to phosphate buffer used in the preparation of other spore types.

The manganese concentration of Mg-C- spores was slightly higher

than other spore types which showed values ranging from 0.1 to 0.14 ug./10^7 spores.

There is some evidence in the results that showed the peptidoglycan of spores were affected by the nature of nutrient depletion. Murrell et al.(1969) examined spore crops of 13 Bacillus species and provided evidence that showed the glucosamine to diaminopimelic acid (D.A.P.) molar ratio fell into six groups having molar ratios of 8, 6, 5, 4, 3 or 2 glucosamine unit to 1 unit D.A.P. The data presented here showed that this ratio is to some extent determined by the nature of the nutrient depletion. Hexosamine/D.A.P. molar ratio was close to unity in Mg-C- spores and was 2.5 for C-, N- and SO_4- spores and about 4 for PO_4- spores. If D.A.P. represents the amount of polypeptide side chains in the polysaccharide backbone of peptidoglycan, then Mg-C- spores obviously possessed the greatest amount of polypeptide side chains. PO_4- spores would have one polypeptide chain on every fifth muramic acid residue (see Murrell et al., 1969). There was no evidence in the results to correlate between the hexosamine/D.A.P. molar ratio and the heat resistance and germination of B. stearothermophilus NCTC 10,003 (mutant) spores.

B. STUDIES ON B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM CARBON DEPLETED CULTURES

In this section of the work, the effect of using different concentrations of medium component (non-depleting) on the properties of spores from carbon depleted culture (C-) were investigated. The

composition of medium used for the preparation of C- spores is shown in Table 17, column 1. All other details for spore preparation were similar to those described in Section 2.F. and 2.G.

(a) The Effect Of Calcium Concentration In Sporulation Medium On The Properties Of Spores Prepared From Carbon Depleted Cultures (C-)

C- spores were prepared from media containing calcium concentrations ranging from 1.0×10^{-6} to 1.4×10^{-4} M. and the properties of these spores determined.

The heat inactivation curves of C- spores prepared from media with different calcium concentrations are shown in Figure 40. D_1, D_2, D_3 and %(R) values were determined from the survivor curves and the results are shown in Table 23. The most heat resistant spores were those prepared from medium with 1.4×10^{-4} M. Ca^{2+} (D_1=10 mn., D_2=168 mn., D_3=115 mn.; %(R)=50.5%) while the most sensitive spores were those from 1.0×10^{-6} M. Ca^{2+} ($D_1 \sim$ 1 mn., D_2=29 mn., D_3=3 mn.; %(R)=8.6%). Spores from 1.0×10^{-4} M. Ca^{2+} medium had identical D_2 value as those from 1.0×10^{-5} M. Ca^{2+} medium. However, D_1 value of spores from 1.0×10^{-4} M. Ca^{2+} was 14 mn. as opposed to $D_1 \sim$ 1 mn. of spores from 1.0×10^{-5} M. Ca^{2+}. The %(R) value was 25.8% and 4.4% in spores from 1.0×10^{-4} M. Ca^{2+} and 1.0×10^{-5} M. Ca^{2+} medium respectively. The results indicated that high medium calcium increased either the "D" or %(R) value or both. The effect of different calcium concentrations in the sporulation medium on the germination of C- spores is shown in

FIGURE 40

HEAT INACTIVATION OF SPORES OBTAINED FROM CARBON DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

All concentrations in molar.

□—□ 1.4×10^{-4} M.

▲—▲ 1.0×10^{-4} M.

▽—▽ 1.0×10^{-5} M.

◆—◆ 1.0×10^{-6} M.

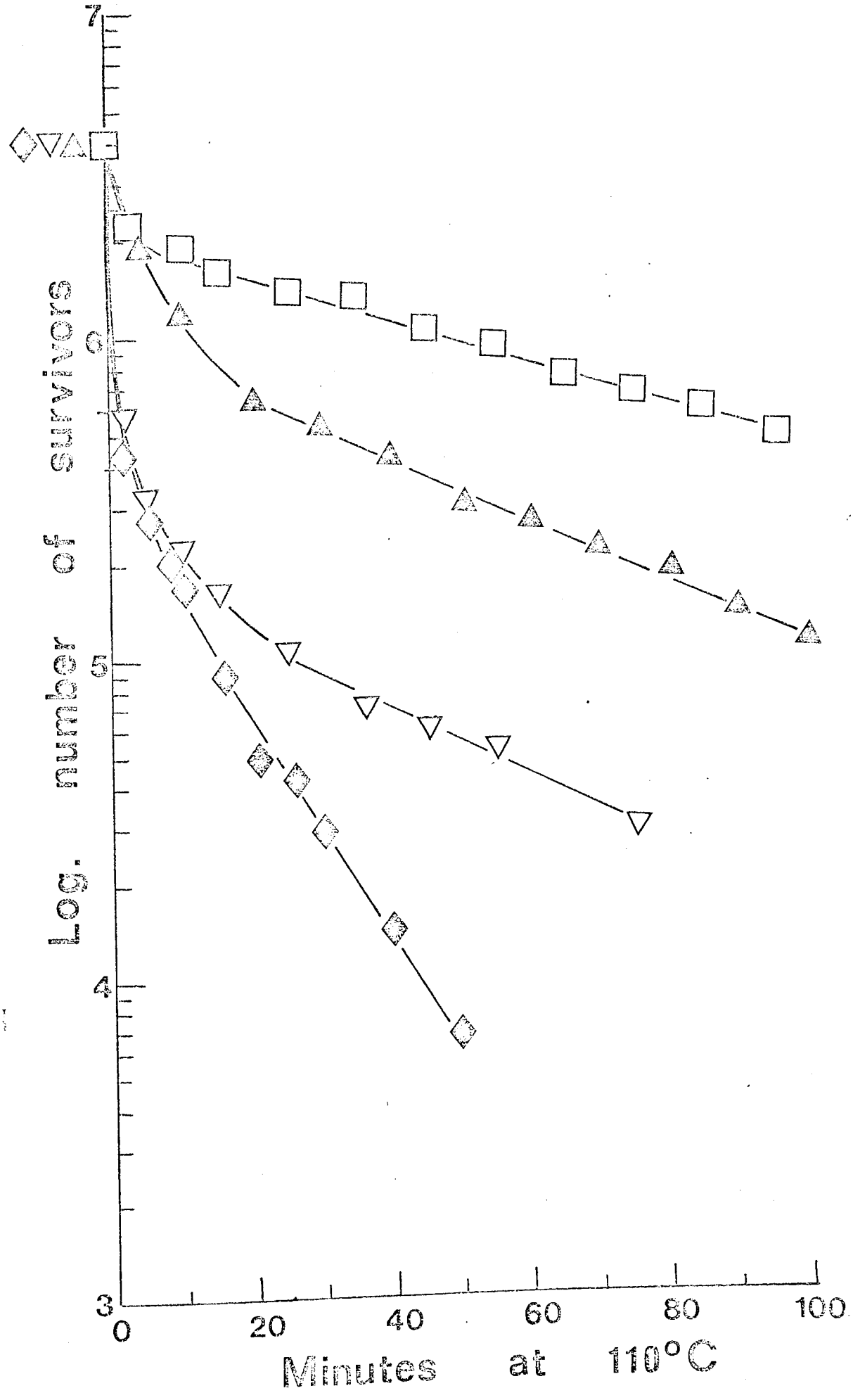

Log. number of survivors
7
6
5
4
3
0
20
40
60
80
100
Minutes at 110°C

TABLE 23

THE EFFECT OF CALCIUM CONCENTRATION IN THE SPORULATION MEDIUM ON THE PROPERTIES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM CARBON DEPLETED CULTURES

	CALCIUM CONCENTRATION (IN MOLAR)			
	1.4×10^{-4}	1.0×10^{-4}	1.0×10^{-5}	1.0×10^{-6}
D.P.A. ($\mu g./10^7$ spores)	1.7250	1.6753	0.9870	0.9520
Calcium ($\times 10^{-1}$ $\mu g./10^7$ spores)	4.4000	3.7500	1.9500	1.6100
$\frac{Ca}{D.P.A.}$ molar ratio	1.06	0.94	0.82	0.71
Magnesium ($\times 10^{-1}$ $\mu g./10^7$ spores)	0.5500	0.7358	5.8110	18.5390
$\frac{Mg}{Ca}$ molar ratio	0.210	0.330	4.970	19.190
Manganese ($\times 10^{-1}$ $\mu g./10^7$ spores)	2.3070	1.2850	0.6200	0.7022
% germination (after 30 mn. at 60°C)	24.0	28.0	51.0	62.0
Heat inactivation at 110°C: D_1 in mn.	∿10	∿14	∿1	∿1
D_2 in mn.	168	102	104	29
D_3 in mn.	115	44	5	3
% (R)	50.5	25.8	4.4	8.6

FIGURE 41

GERMINATION OF SPORES OBTAINED FROM CARBON DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

All concentrations in molar.

△—△ 1.4×10^{-4} M.

◇—◇ 1.0×10^{-4} M.

□—□ 1.0×10^{-5} M.

△—△ 1.0×10^{-6} M.

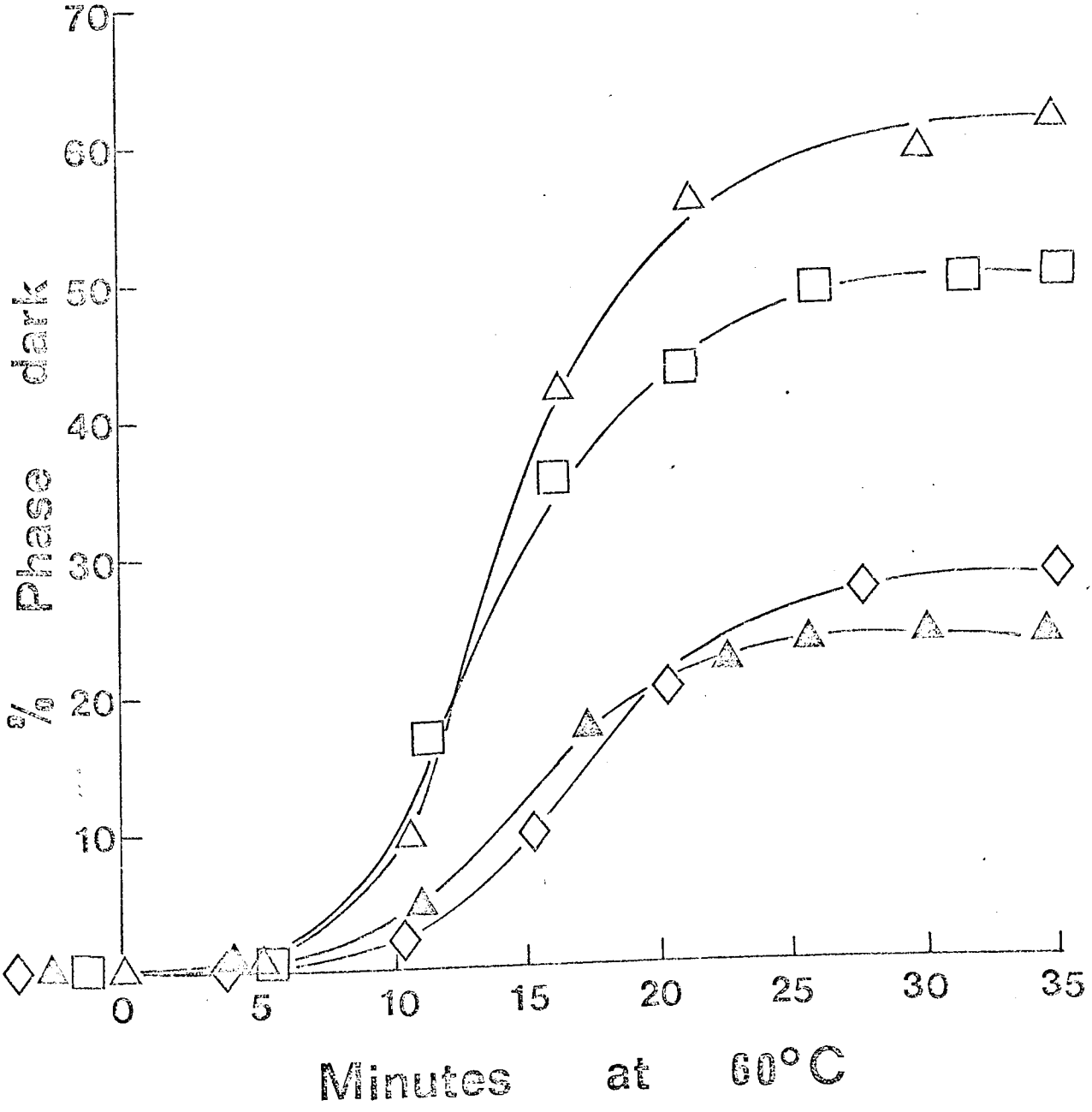

70
60
50
40
30
20
10
% phase dark
0
5
10
15
20
25
30
35
Minutes at 60° C

Figure 41. There is evidence that high medium calcium promoted the formation of dormant spores. Spores from the medium with 1.0×10^{-4} M. or 1.4×10^{-4} M. Ca^{2+} germinated less quickly and less extensively than spores prepared from medium with 1.0×10^{-5} M. or 1.0×10^{-6} M. Ca^{2+}. The percentage of germinated spores after 30 minutes incubation was determined and the results are shown in Table 23. Stastna and Vinter (1970) found that B. cereus spores grown so as to contain large amounts of Ca-D.P.A. (Ca-D.P.A. rich spores) did not germinate in complex medium with high concentration of calcium (0.5 M. $CaCl_2$) whereas Ca-D.P.A. deficient spores germinated optimally in such medium. Ca-D.P.A. rich spores germinated optimally in synthetic medium low in Ca^{2+} (0.1 to 0.4 M.). The same results were obtained when other divalent cations such as Mg^{2+}, Ba^{2+} or Sr^{2+} were used. In the present study, the germination agar contained no added cations. Since complex medium (glucose-tryptone agar) was used, the contaminating level of cations was likely to be moderate.

Spores were also analysed for D.P.A., Ca^{2+}, Mg^{2+} and Mn^{2+} concentrations as described in Section 2.K. to 2.M. and the results are shown in Table 23. There is some evidence in the results to suggest that the calcium and D.P.A. concentrations of spores were dependent upon the calcium concentration in the sporulation medium. Spores with high calcium and D.P.A. concentrations were those from medium with 1.4×10^{-4} M. or 1.0×10^{-4} M. calcium and these spores were highly resistant. With the exception of spores from 1.0×10^{-6} M. Ca^{2+} medium, the Ca/D.P.A. molar ratio was close to unity. Spores from medium low in calcium concentration tended to

have higher magnesium concentrations. The Mg/Ca molar ratio was highest with spores prepared from 1.0×10^{-6} M. Ca^{2+} medium and these spores were extremely heat sensitive. This finding is in agreement with that of Murrell and Warth (1965). They showed that the Mg/Ca molar ratio increased significantly with decrease in the heat resistance ($P \ll 0.001$) while the calcium concentration increased significantly with increase in the heat resistance of spores. Spores with lower calcium content were also low in manganese.

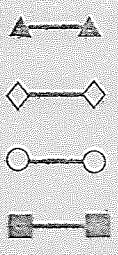
(b) The Effect Of Iron Concentration In Sporulation Medium On The Properties Of Spores Prepared From Carbon Depleted Cultures (C-)

The effect of using different iron concentrations in the media on the heat resistance, germination and chemistry of C- spores are shown in Figures 42 and 43 and Table 24. The data suggested that high medium iron (1.0×10^{-5} M. and above) reduced the heat resistance of C- spores produced. These spores had lower D_1, D_2, D_3 and %(R) values and they were also more dormant than corresponding spores prepared from medium with lower iron concentration. D.P.A. and calcium concentration of spores were higher in spores with higher heat resistance. D.P.A./Ca molar ratio was closed to unity in all spores. Spores with higher D.P.A. and calcium concentration, on the other hand had lower magnesium concentration and Mg/Ca molar ratio, these spores also germinated more rapidly and extensively (Figure 43). The data with manganese content were less conclusive; spores from higher iron medium appeared to

FIGURE 42

HEAT INACTIVATION OF SPORES OBTAINED FROM CARBON DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) CONTAINING DIFFERENT CONCENTRATIONS OF IRON

All concentrations in molar.



1.0×10^{-8} M.

1.0×10^{-7} M.

1.0×10^{-5} M.

2.0×10^{-5} M.

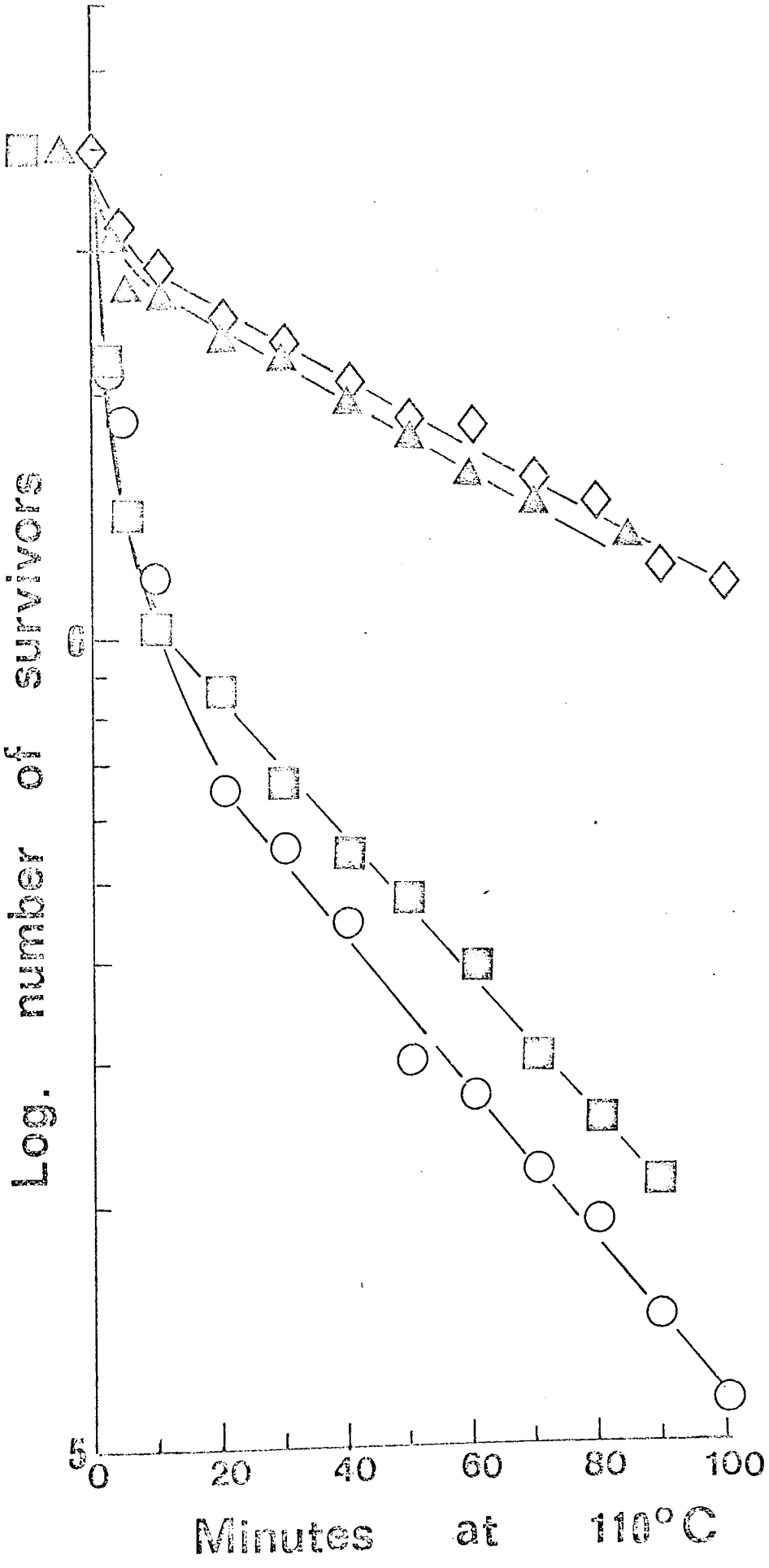

Log. number of survivors
6
5
0
20
40
60
80
100
Minutes at 110°C

FIGURE 43

GERMINATION OF SPORES OBTAINED FROM CARBON DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) CONTAINING DIFFERENT CONCENTRATIONS OF IRON

All concentrations in molar.

○—○ 1.0×10^{-8} M.

△—△ 1.0×10^{-7} M.

□—□ 1.0×10^{-5} M.

▽—▽ 2.0×10^{-5} M.

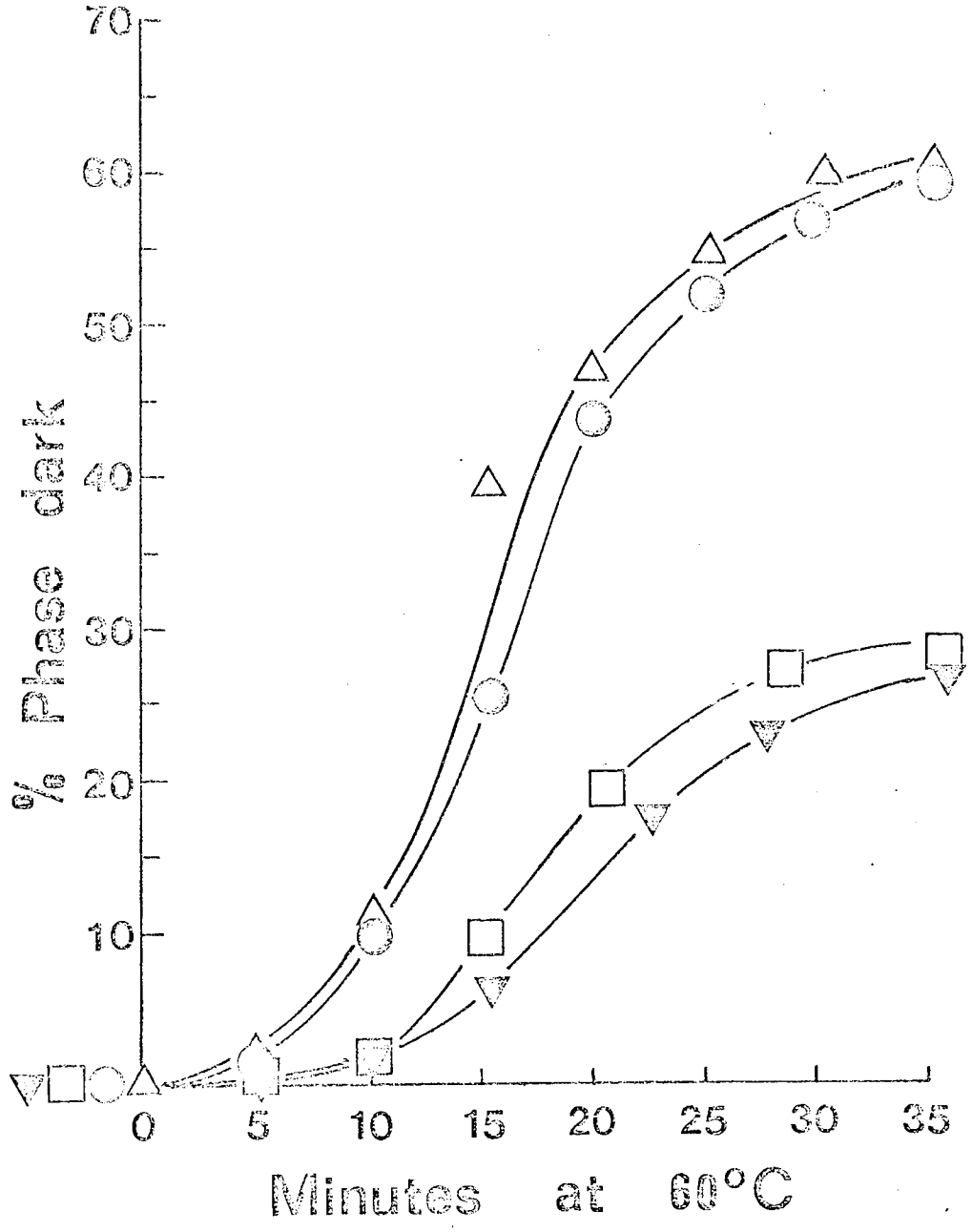

70
60
50
40
30
20
10
% Phase dark
0
5
10
15
20
25
30
35
Minutes at 60°C

TABLE 24

THE EFFECT OF IRON CONCENTRATION IN THE SPORULATION MEDIUM ON THE PROPERTIES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM CARBON DEPLETED CULTURES

	IRON CONCENTRATION (IN MOLAR)			
	2.0×10^{-5}	1.0×10^{-5}	1.0×10^{-7}	1.0×10^{-8}
D.P.A. ($\mu g./10^7$ spores)	1.4320	1.6753	1.7470	1.9440
Calcium ($\times 10^{-1}$ $\mu g./10^7$ spores)	3.5000	3.7500	4.1020	5.0480
$\frac{Ca}{D.P.A.}$ molar ratio	1.02	0.94	0.98	1.09
Magnesium ($\times 10^{-1}$ $\mu g./10^7$ spores)	0.6510	0.7351	0.1747	0.1805
$\frac{Mg}{Ca}$ molar ratio	0.31	0.33	0.07	0.06
Manganese ($\times 10^{-1}$ $\mu g./10^7$ spores)	2.2075	1.2850	1.2200	1.3500
% germination (after 30 mn. at 60°C)	25.0	28.0	58.0	60.0
Heat inactivation at 110°C: D_1 in mn.	≤6	≤10	≤44	≤44
D_2 in mn.	113	102	254	254
D_3 in mn.	59	44	230	221
% (R)	32.8	25.8	77.0	74.5

have a higher concentration of manganese.

(c) The Effect Of Manganese Concentration In Sporulation Medium On The Properties Of Spores Prepared From Carbon Depleted Cultures (C-)

The effect of different manganese concentrations in the sporulation medium on the heat resistance, germination and chemistry of C- spores are shown in Figures 44, 45 and Table 25. The pattern of heat inactivation, germination, D.P.A., calcium, magnesium and Mg/Ca molar ratio were similar to those obtained from C- spores prepared with different medium irons. The manganese concentration of C- spores was typically high in spores prepared from media containing high concentration of manganese (1.0×10^{-4} M. and above)

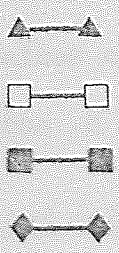
(d) The Effect Of Magnesium Concentration In Sporulation Medium On The Properties Of Spores Prepared From Carbon Depleted Cultures (C-)

The effect of different magnesium concentrations in the sporulation medium on the heat resistance and chemistry of C- spores is shown in Figure 46 and Table 26. The magnesium and manganese concentrations in C- spores were high in spores prepared from medium containing exceptionally high concentration of magnesium (5.0×10^{-3} M.). However, different magnesium conc. in the sporulation medium did not appear to affect the heat resistance of C- spores produced. This is in agreement with the results of Friesen and Anderson (1974). These workers showed that reduction of medium magnesium did not significantly change the heat resistance of the resulting spores.

FIGURE 44

HEAT INACTIVATION OF SPORES OBTAINED FROM CARBON DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) CONTAINING DIFFERENT CONCENTRATIONS OF MANGANESE

All concentrations in molar.



1.0×10^{-6} M.

1.0×10^{-5} M.

1.0×10^{-4} M.

2.0×10^{-4} M.

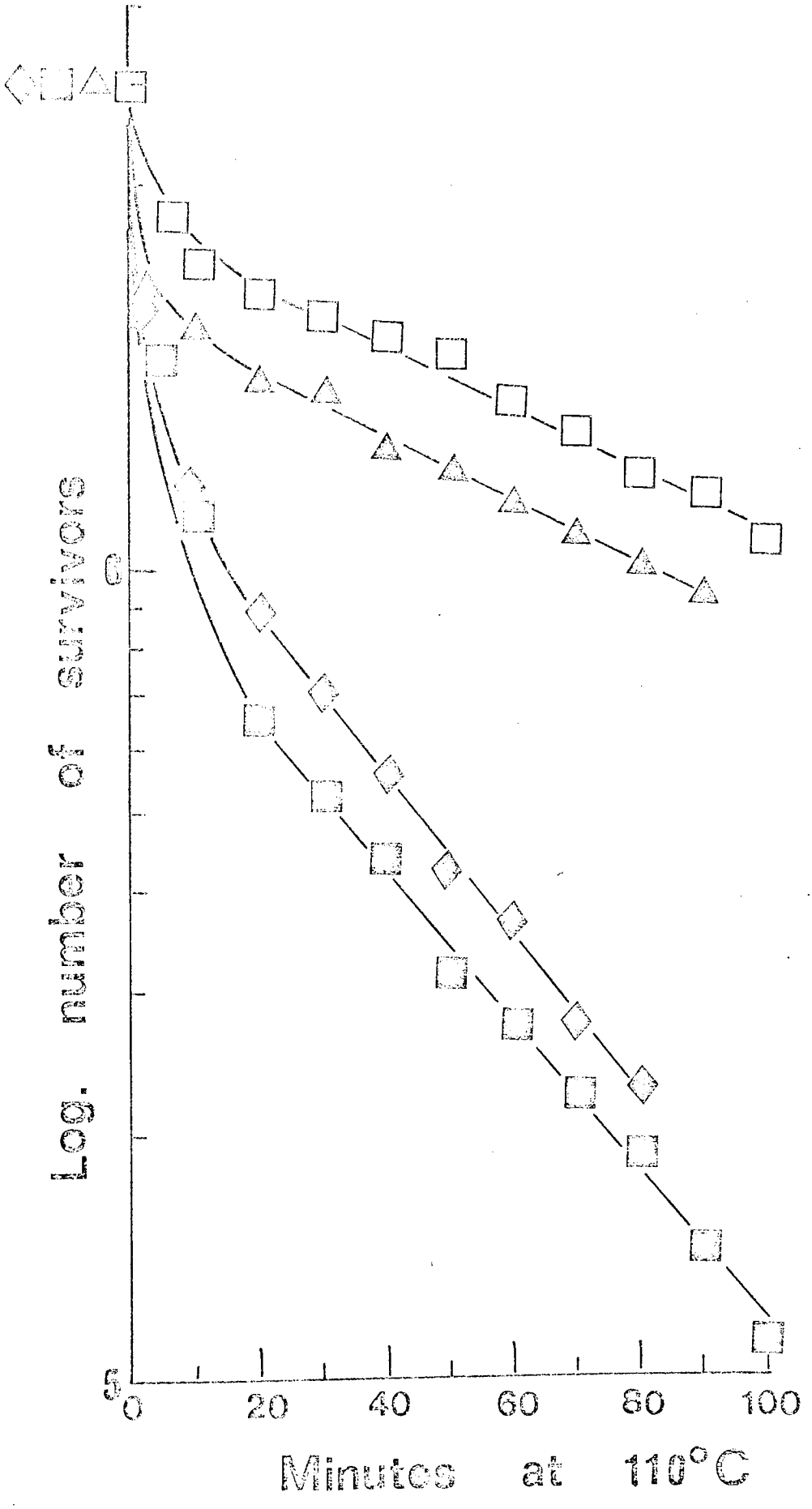

Log. number of survivors
6
5
0
20
40
60
80
100
Minutes at 110°C

FIGURE 45

GERMINATION OF SPORES OBTAINED FROM CARBON DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) CONTAINING DIFFERENT CONCENTRATIONS OF MANGANESE

All concentrations in molar.

◇——◇ 1.0×10^{-6} M.

●——● 1.0×10^{-5} M.

△——△ 1.0×10^{-4} M.

▽——▽ 2.0×10^{-4} M.

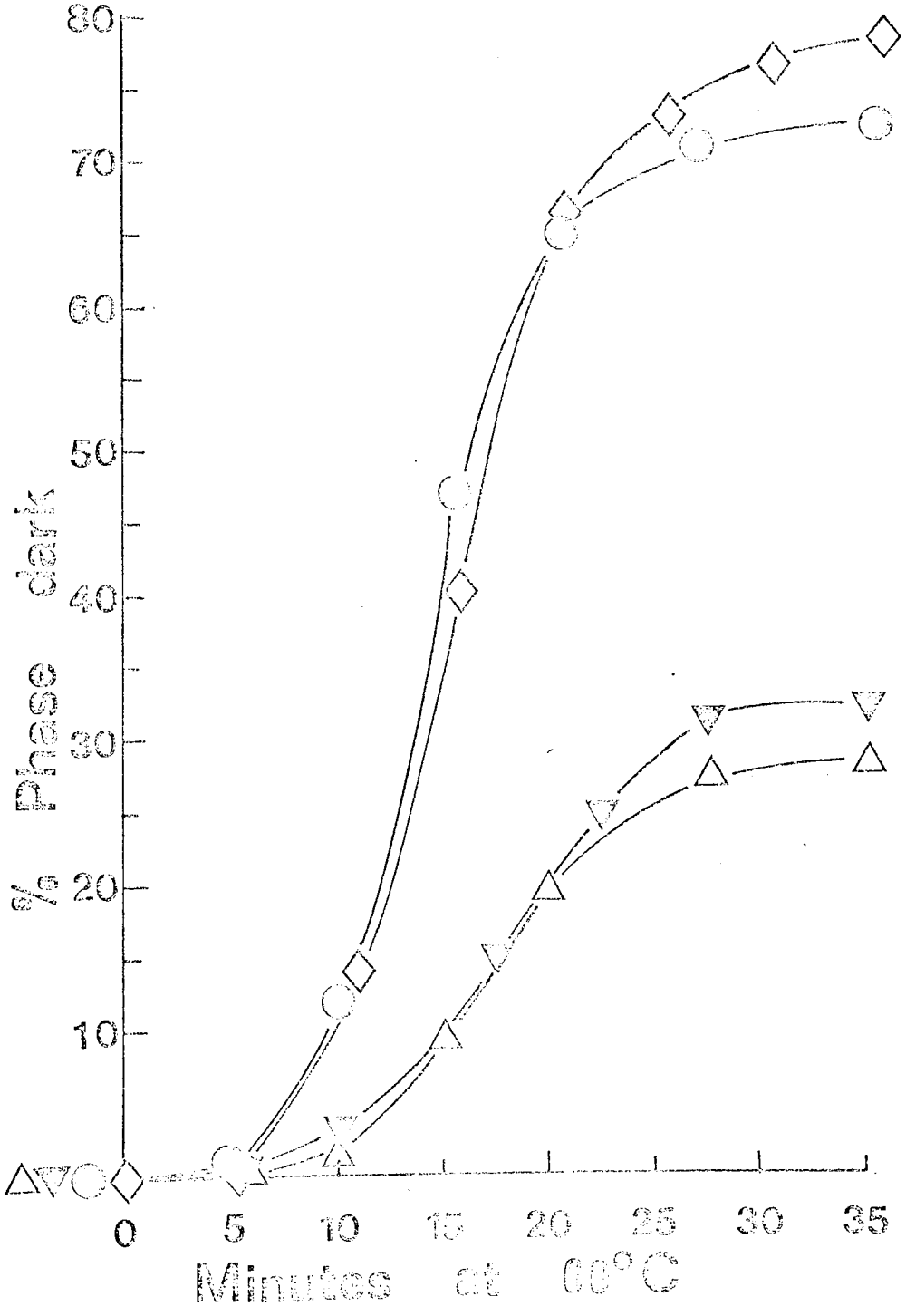

80
70
60
50
40
30
20
10
0
% Phase dark
5
10
15
20
25
30
35
Minutes at 60°C

TABLE 25

THE EFFECT OF MANGANESE CONCENTRATION IN THE SPORULATION MEDIUM ON THE PROPERTIES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM CARBON DEPLETED CULTURES

	MANGANESE CONCENTRATION (IN MOLAR)			
	2.0×10^{-4}	1.0×10^{-4}	1.0×10^{-5}	1.0×10^{-6}
D.P.A. ($\mu g./10^7$ spores)	1.1386	1.6753	1.6370	1.5921
Calcium ($\times 10^{-1}$ $\mu g./10^7$ spores)	3.0100	3.7500	4.7200	4.5000
$\frac{Ca}{D.P.A.}$ molar ratio	1.10	0.94	1.20	1.18
Magnesium ($\times 10^{-1}$ $\mu g./10^7$ spores)	0.6500	0.7358	0.3430	0.3740
$\frac{Mg}{Ca}$ molar ratio	0.36	0.33	0.12	0.18
Manganese ($\times 10^{-1}$ $\mu g./10^7$ spores)	15.9450	1.2850	0.6400	0.2000
% germination (after 30 mn. at 60°C)	32.5	28.0	72.0	78.0
Heat inactivation at 110°C: D_1 in mn.	≤14	≤14	≤29	≤18
D_2 in mn.	108	102	261	262
D_3 in mn.	55	44	219	189
% (R)	35.4	25.8	69.4	53.5

FIGURE 46

HEAT INACTIVATION OF SPORES OBTAINED FROM CARBON DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) CONTAINING DIFFERENT CONCENTRATIONS OF MAGNESIUM

▽——▽ 5.0×10^{-3} Molar magnesium

■——■ 5.0×10^{-4} Molar magnesium

Note: Each point represents the average of at least two separate determinations.

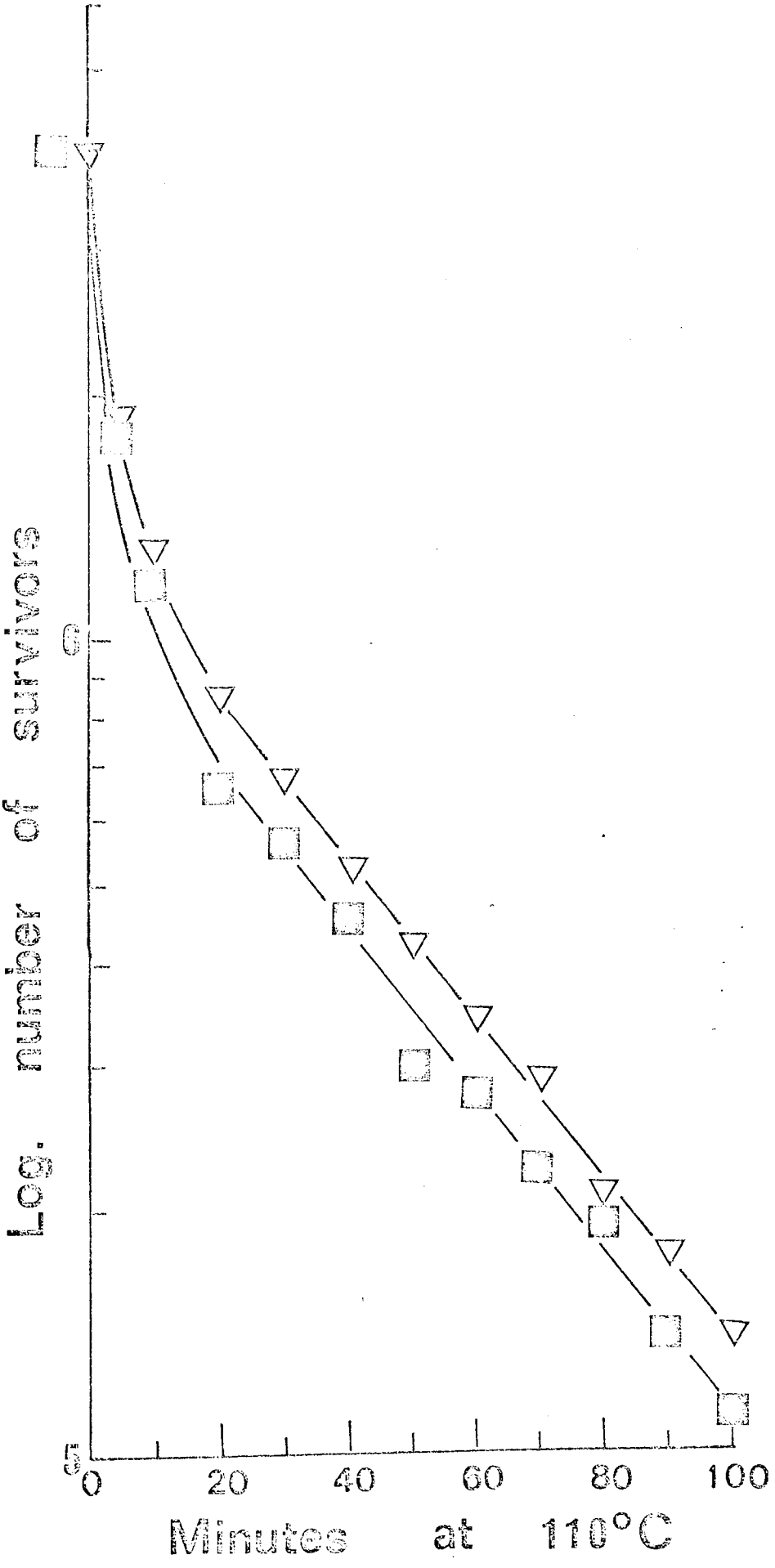

Log. number of survivors
6
5
0
20
40
60
80
100
Minutes at 110°C

TABLE 26

THE EFFECT OF MAGNESIUM CONCENTRATION IN THE SPORULATION MEDIUM ON THE PROPERTIES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM CARBON DEPLETED CULTURES

	MAGNESIUM CONCENTRATION (IN MOLAR)	
	5.0×10^{-3}	5.0×10^{-4}
D.P.A. ($\mu g./10^7$ spores)	1.5627	1.6753
Calcium ($\times 10^{-1}$ $\mu g./10^7$ spores)	4.0010	3.7500
$\frac{Ca}{D.P.A.}$ molar ratio	1.07	0.94
Magnesium ($\times 10^{-1}$ $\mu g./10^7$ spores)	77.1950	0.7358
$\frac{Mg}{Ca}$ molar ratio	32.16	0.33
Manganese ($\times 10^{-1}$ $\mu g./10^7$ spores)	7.7480	1.2850
Heat inactivation at 110°C: D_1 (in mn.)	$\simeq 21$	$\simeq 14$
D_2 (in mn.)	101	102
D_3 (in mn.)	52	44
% (R)	32.8	25.8

(e) The Effect Of Phosphate Concentration In Sporulation Medium On The Properties Of Spores Prepared From Carbon Depleted Cultures (C-)

The effect of different phosphate concentration in the sporulation medium on the heat resistance and chemistry of C- spores is shown in Figure 47 and Table 27. Interpretation of these results must take into consideration the fact that the sporulation medium of spores from lower phosphate medium was buffered with HEPES buffer whereas those from higher phosphate medium was buffered with phosphate buffer. Decrease in the medium phosphate appeared to promote the accumulation of magnesium and manganese in C- spores. The percentage of heat resistant spores was increased but D_2 value was only slightly lowered with the decrease of phosphate level in sporulation medium.

(f) The Effect Of Sulphate Concentration In Sporulation Medium On The Properties Of Spores Prepared From Carbon Depleted Cultures (C-)

The effect of different sulphate concentrations in the sporulation medium on the heat resistance and chemistry of C- spores is shown in Figure 48 and Table 28. The data show that lowering of medium sulphate increased the heat resistance of spores produced. The concentration of calcium and dipicolinic acid in spores were increased with increase in spore heat resistance. The Mg/Ca molar ratio was decreased slightly. Changes in the sulphate level of medium did not appreciably affect the concentration of manganese in spores.

FIGURE 47

HEAT INACTIVATION OF SPORES OBTAINED FROM CARBON DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) CONTAINING DIFFERENT CONCENTRATIONS OF PHOSPHATE

△——△ 2.40×10^{-4} M. phosphate

▽——▽ 2.49×10^{-2} M. phosphate

Note: Each point represents the average of at least two separate determinations.

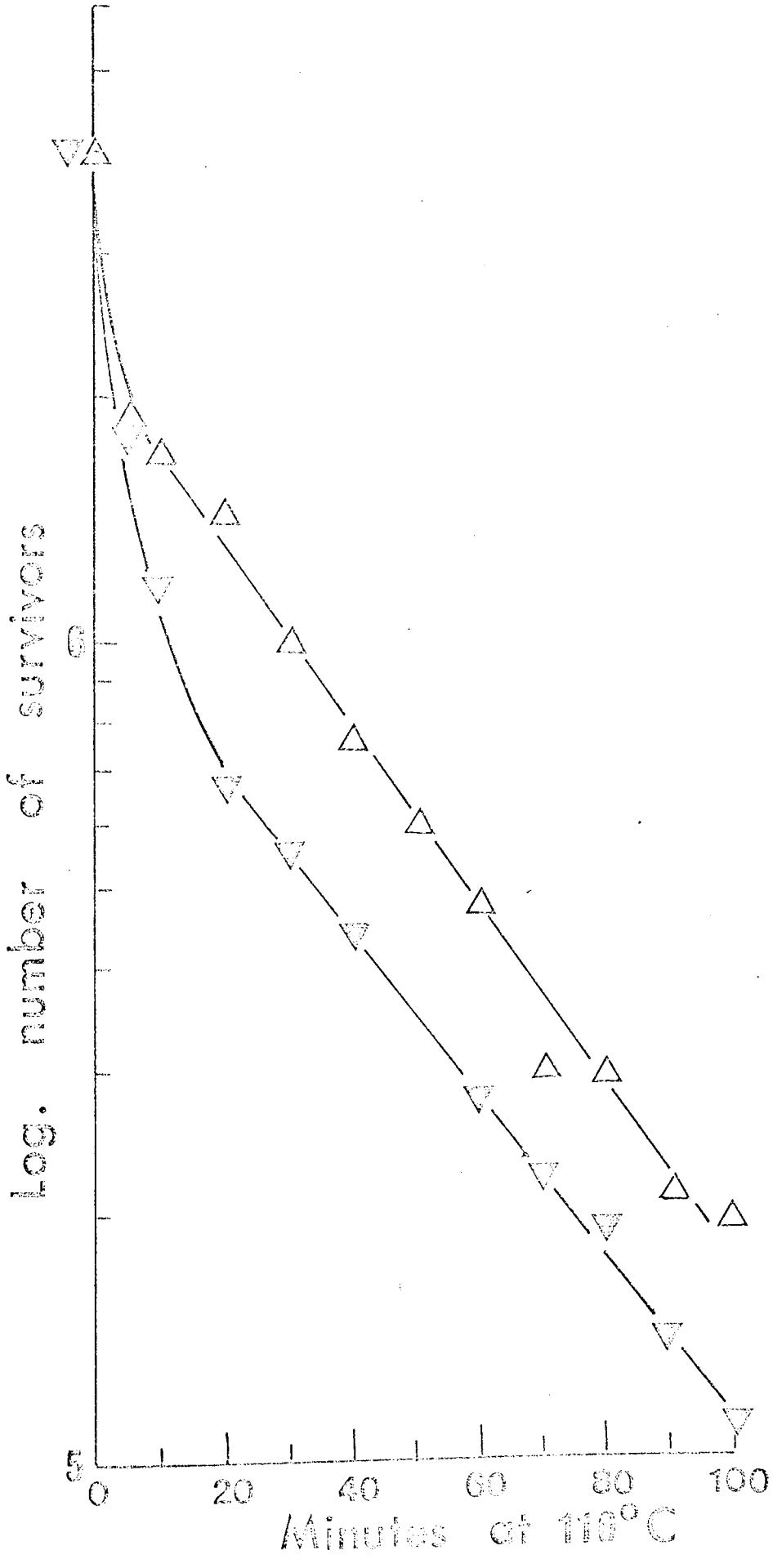

Log. number of survivors
7
6
5
0
20
40
60
80
100
Minutes at 118°C

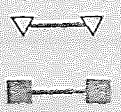
TABLE 27

THE EFFECT OF PHOSPHATE CONCENTRATION IN THE SPORULATION MEDIUM ON THE PROPERTIES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM CARBON DEPLETED CULTURES

	PHOSPHATE CONCENTRATION (IN MOLAR)	
	2.49×10^{-2}	2.40×10^{-4} (HEPES BUFFERED)
D.P.A. ($\mu g./10^7$ spores)	1.6753	1.6156
Calcium ($\times 10^{-1}$ $\mu g./10^7$ spores)	3.7500	3.7480
$\frac{Ca}{D.P.A.}$ molar ratio	0.94	0.97
Magnesium ($\times 10^{-1}$ $\mu g./10^7$ spores)	0.7358	5.4010
$\frac{Mg}{Ca}$ molar ratio	0.33	2.40
Manganese ($\times 10^{-1} \mu g./10^7$ spores)	1.2850	2.8000
Heat inactivation at 110°C: D_1 in mn.	≤ 14	≤ 20
D_2 in mn.	102	91.2
D_3 in mn.	44	67
% (R)	25.8	55.6

FIGURE 48

HEAT INACTIVATION OF SPORES OBTAINED FROM CARBON DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) CONTAINING DIFFERENT CONCENTRATIONS OF SULPHATE



▽—▽ 4.73×10^{-5} M. sulphate

■—■ 6.10×10^{-4} M. sulphate

Note: Each point represents the average of at least two separate determinations.

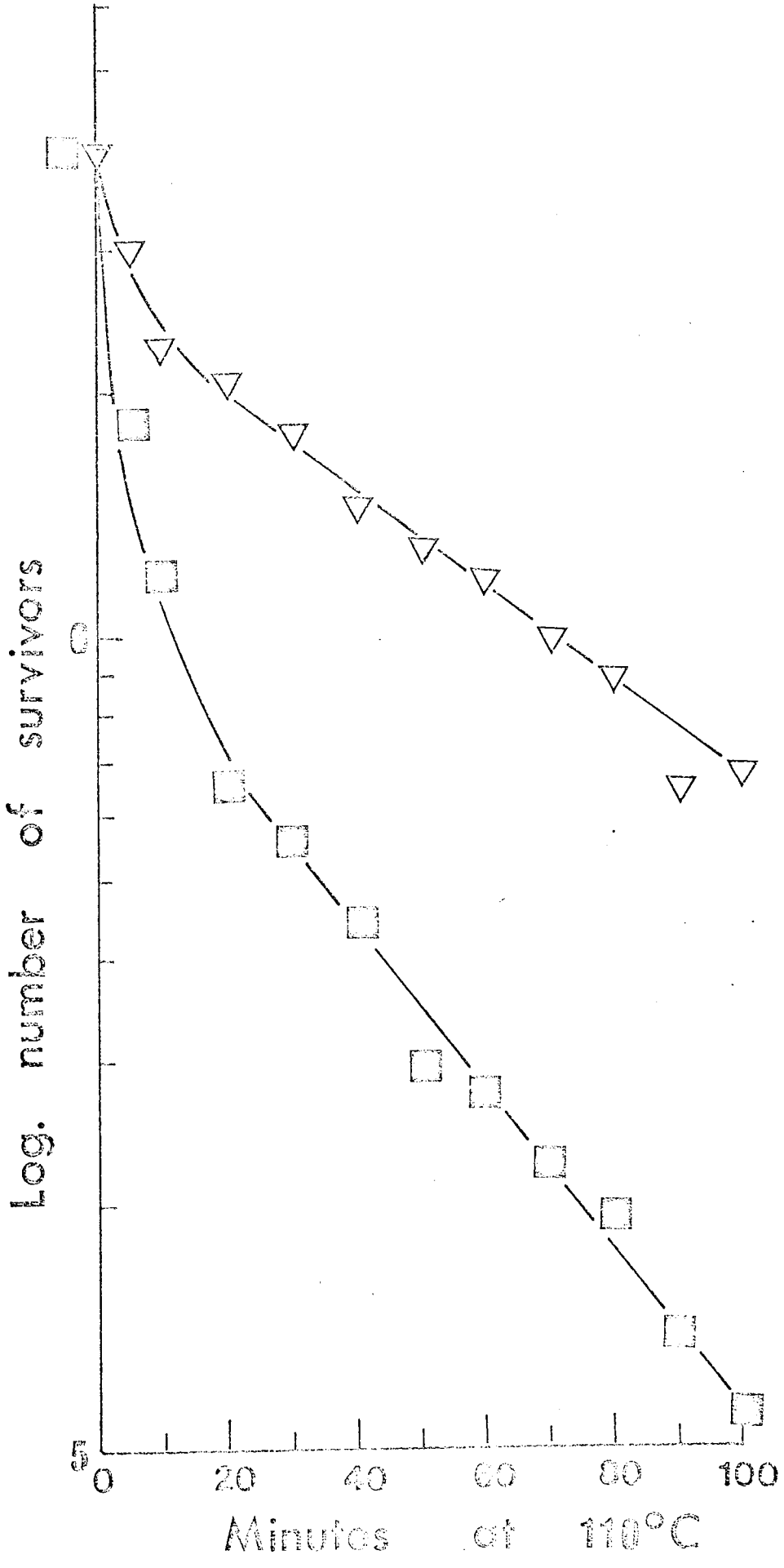

log. number of survivors
6
5
0
20
40
60
80
100
Minutes at 110°C

TABLE 28

THE EFFECT OF SULPHATE CONCENTRATION IN THE SPORULATION MEDIUM ON THE PROPERTIES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM CARBON DEPLETED CULTURES

	SULPHATE CONCENTRATION (IN MOLAR)	
	6.10×10^{-4}	4.73×10^{-5}
D.P.A. ($\mu g./10^7$ spores)	1.6753	1.7500
Calcium ($\times 10^{-1}$ $\mu g./10^7$ spores)	3.7500	4.6000
$\frac{Ca}{D.P.A.}$ molar ratio	0.94	1.10
Magnesium ($\times 10^{-1}$ $\mu g./10^7$ spores)	0.74	0.64
$\frac{Mg}{Ca}$ molar ratio	0.33	0.23
Manganese ($\times 10^{-1}$ $\mu g./10^7$ spores)	1.2850	1.3800
Heat inactivation at 110°C: D_1 in mn.	≤14	≤34
D_2 in mn.	102	173
D_3 in mn.	44	140
% (R)	25.8	65.7

C. STUDIES ON B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM NITROGEN DEPLETED CULTURES

Nutrient depletion studies of various spore kinds showed the extraordinary heat resistance of spores prepared from nitrogen depleted cultures (N-) (see Section 6.A.). Consequently, these spores were chosen for further studies and the effect of various concentrations of medium calcium on the properties of spores were determined. Calcium is well noted for its influence on spore heat resistance (Slepecky and Foster, 1959; Friesen and Anderson, 1974).

The composition of the medium used to prepare N- spores is shown in Table 17, column 2. Spores were prepared from media with the following concentrations of calcium: 1.4×10^{-4} M., 1.0×10^{-4} M., 5.0×10^{-5} M. and 1.0×10^{-5} M. The method used for spore cultivation and preparation have been described in Section 2.F. and 2.G. respectively.

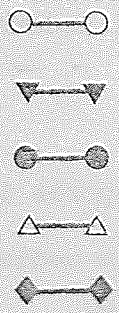
(a) The Effect Of Calcium Concentration In Sporulation Medium On The Heat Resistance Of Spores Prepared From Nitrogen Depleted Cultures (N-)

The inactivation curves of N- spores prepared from media with different calcium concentrations are shown in Figure 49 to 52. In each case, heating temperatures of 105°, 110°, 115°, 118° and 121°C were used. The inactivation curve was triphasic at temperature of 118°C and above. In addition to the second exponential decline phase described previously, a third phase of rapid inactivation was observed.

FIGURE 49

HEAT INACTIVATION OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM NITROGEN DEPLETED CULTURES CONTAINING 1.4×10^{-4} M. CALCIUM.

INACTIVATION TEMPERATURE (°C)



105°

110°

115°

118°

121°

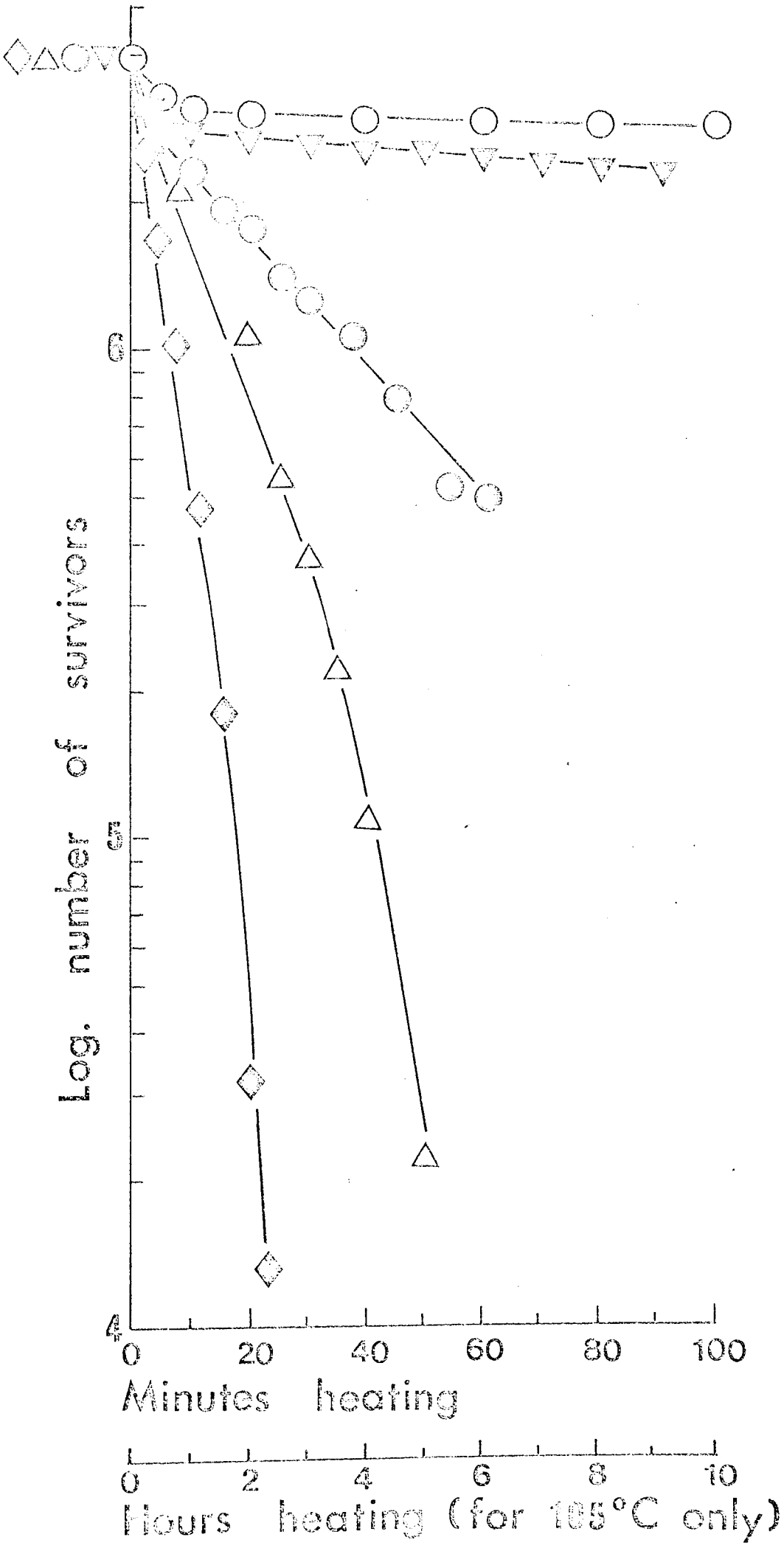
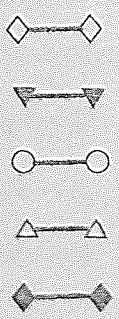

Log. number of survivors
6
5
4
0
20
40
60
80
100
Minutes heating
0
2
4
6
8
10
Hours heating (for 105°C only)

FIGURE 50

HEAT INACTIVATION OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM NITROGEN DEPLETED CULTURES CONTAINING 1.0×10^{-4} M. CALCIUM

INACTIVATION TEMPERATURE (°C)



105°

110°

115°

118°

121°

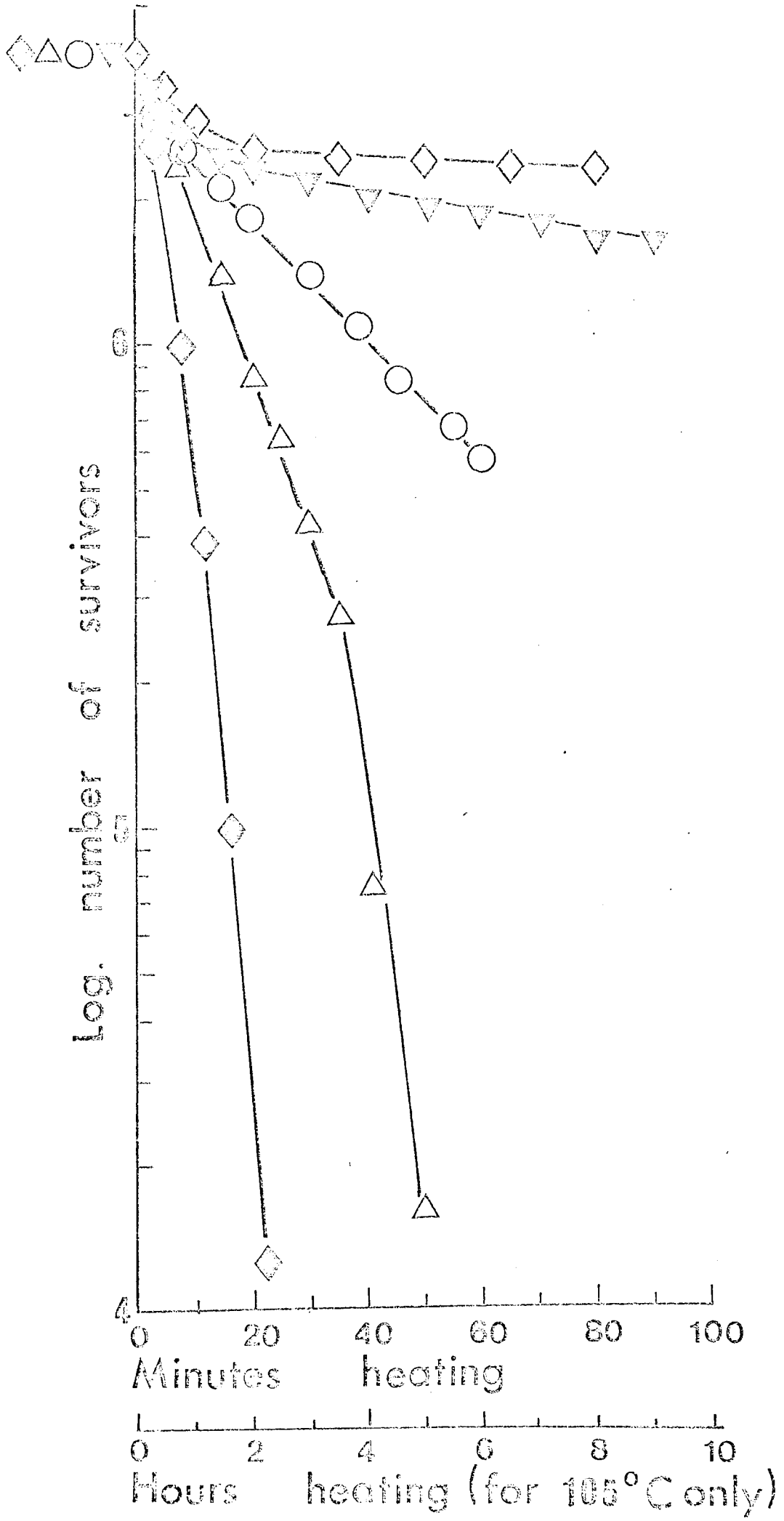
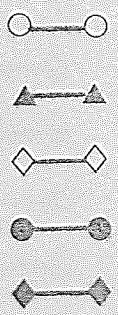

Log. number of survivors
6
5
4
0
20
40
60
80
100
Minutes heating
0
2
4
6
8
10
Hours heating (for 105° C only)

FIGURE 51

HEAT INACTIVATION OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM NITROGEN DEPLETED CULTURES CONTAINING 5.0×10^{-5} M. CALCIUM

INACTIVATION TEMPERATURE (°C)



105°

110°

115°

118°

121°

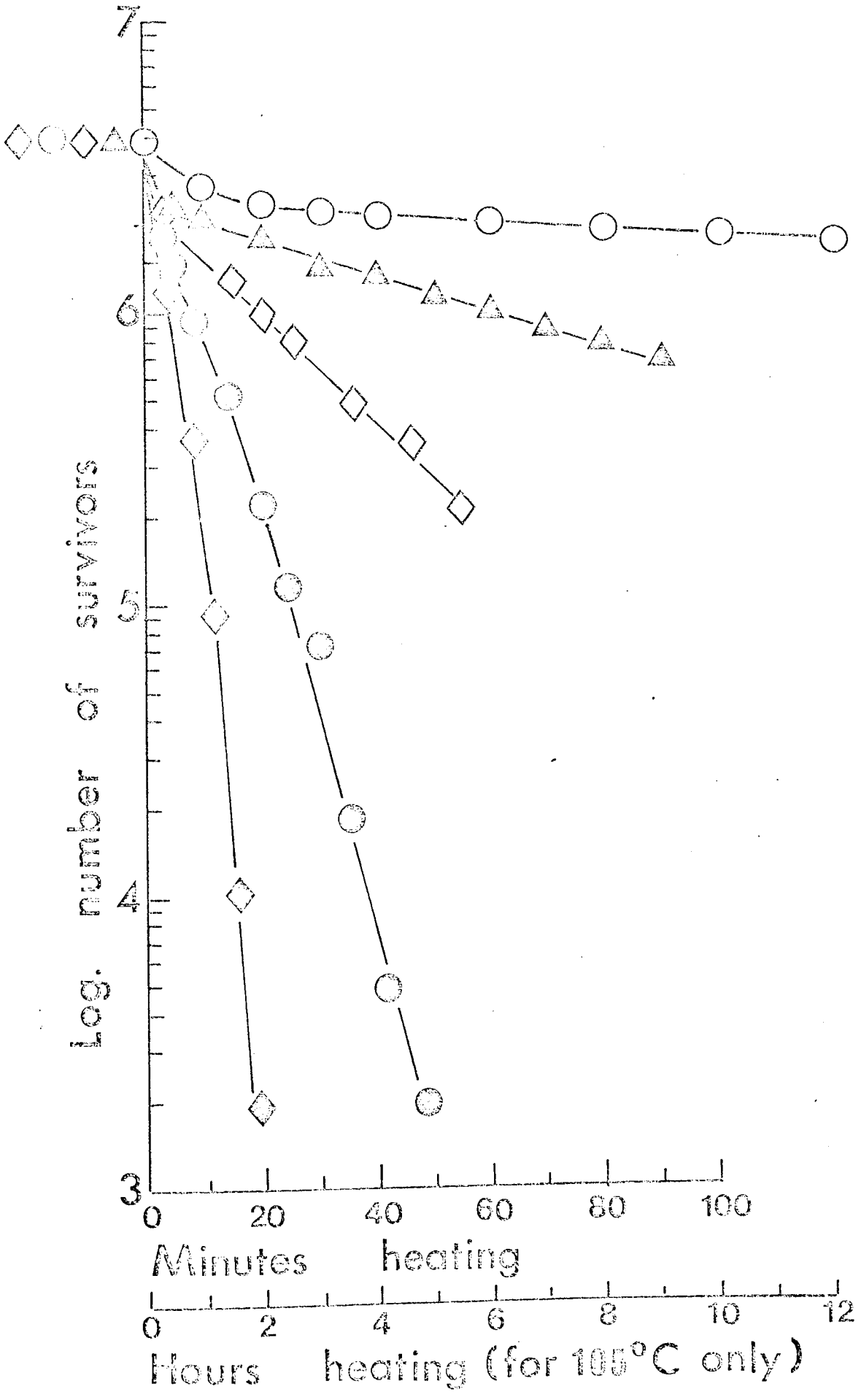
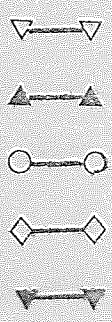

7
6
5
4
3
log. number of survivors
0
20
40
60
80
100
Minutes heating
0
2
4
6
8
10
12
Hours heating (for 105°C only)

FIGURE 52

HEAT INACTIVATION OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM NITROGEN DEPLETED CULTURES CONTAINING 1.0×10^{-5} M. CALCIUM.

INACTIVATION TEMPERATURE (°C)



105°

110°

115°

118°

121°

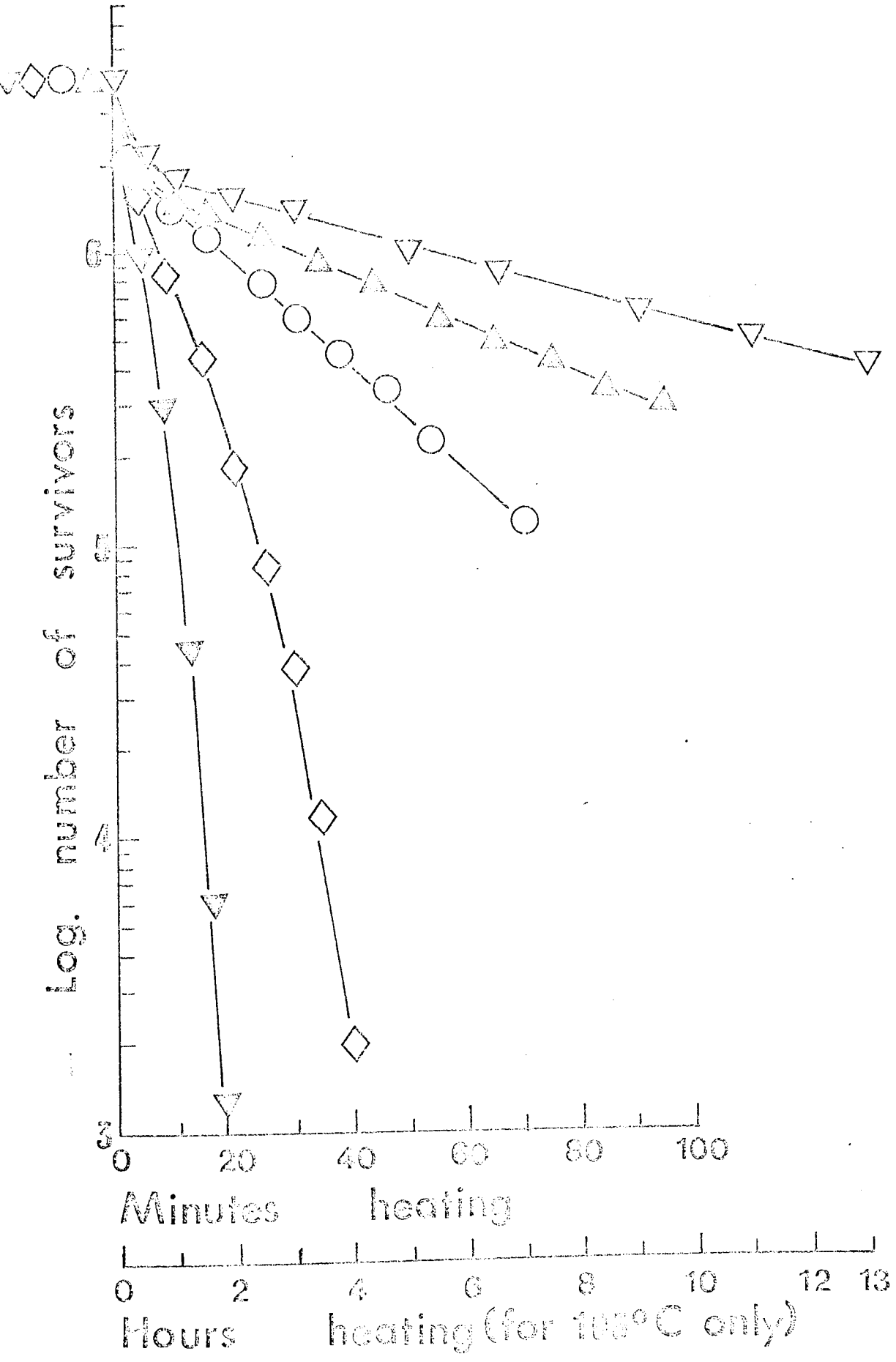

log. number of survivors
6
5
4
3
0
20
40
60
80
100
Minutes heating
0
2
4
6
8
10
12
13
Hours heating (for 100°C only)

TABLE 29

THE EFFECT OF CALCIUM CONCENTRATION IN THE SPORULATION MEDIUM ON THE PROPERTIES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM NITROGEN DEPLETED CULTURES

	CALCIUM CONCENTRATION (IN MOLAR)			
	1.4×10^{-4}	1.0×10^{-4}	5.0×10^{-5}	1.0×10^{-5}
D.P.A. ($\mu g./10^7$ spores)	1.9182	1.9560	1.8230	1.6950
Calcium ($\times 10^{-1}$ $\mu g./10^7$ spores)	5.7110	5.2000	4.8000	4.2500
$\frac{Ca}{D.P.A.}$ molar ratio	1.24	1.11	1.10	1.05
Magnesium ($\times 10^{-1}$ $\mu g./10^7$ spores)	0.0912	0.2500	0.5211	1.0594
$\frac{Mg}{Ca}$ molar ratio	0.03	0.01	0.18	0.41
Manganese ($\times 10^{-1}$ $\mu g./10^7$ spores)	1.3484	1.4090	1.0420	1.9550
% germination (after 30 mn. at 60°C)	70	75	80	83
Heat inactivation at 110°C: D_1 in mn.	~31	~42	~14	~12
D_2 in mn.	1422	478	171	120
D_3 in mn.	1212	385	132	82
% (R)	72.0	63.0	60.6	45.4

D_1, D_2, D_3 and %(R) values of the various spore kinds were determined at 110°C as described previously and the results are shown in Table 29. There is some evidence in the data that showed the calcium concentration in the medium affected the D_1, D_2, D_3 and %(R) values of N- spores prepared. The heat resistance of spores was increased with increase in the concentration of medium calcium. Since D_2 values can be determined with greater accuracy than other "D" values, this parameter was determined with spores heated at various inactivating temperatures (Figures 49 to 52). The plot of log. D_2 values against the concentrations of medium calcium is shown in Figure 53. A linear relationship between log. D_2 values and the concentration of medium calcium was obtained. The slope of the line was steeper when the heating temperatures were 110°C and below, indicating that the effect of medium calcium concentration on thermal resistance was more pronounced when the heating temperatures were 110°C and below.

Arrhenius plots for the thermal inactivation of N- spores of various kinds are shown in Figure 54. K, the rate constant for the inactivation process was calculated from the D-value (k = 2.303/D). The slope of Arrhenius plots was determined by regression and the activation energy calculated. Transition state theory permits calculation of the free energy of activation (ΔG).

$$\Delta G = 2.303RT \log. \frac{K'T}{kh}$$

R = ideal gas constant

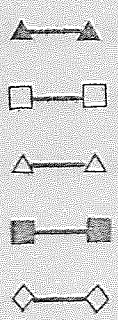
K' = Boltzman constant (1.36×10^{-8} erg deg.$^{-1}$).

h = Planck constant (6.624×10^{-27} ergs).

k = rate constant in sec.$^{-1}$ at absolute temperature T.

FIGURE 53

THE RELATIONSHIP BETWEEN LOG.D_2(AT VARIOUS INACTIVATION TEMPERATURES) AND THE CONCENTRATION OF SPORULATION CALCIUM OF SPORES OBTAINED FROM NITROGEN DEPLETED CULTURES OF *B. STEAROTHERMOPHILUS* NCTC 10,003 (MUTANT)



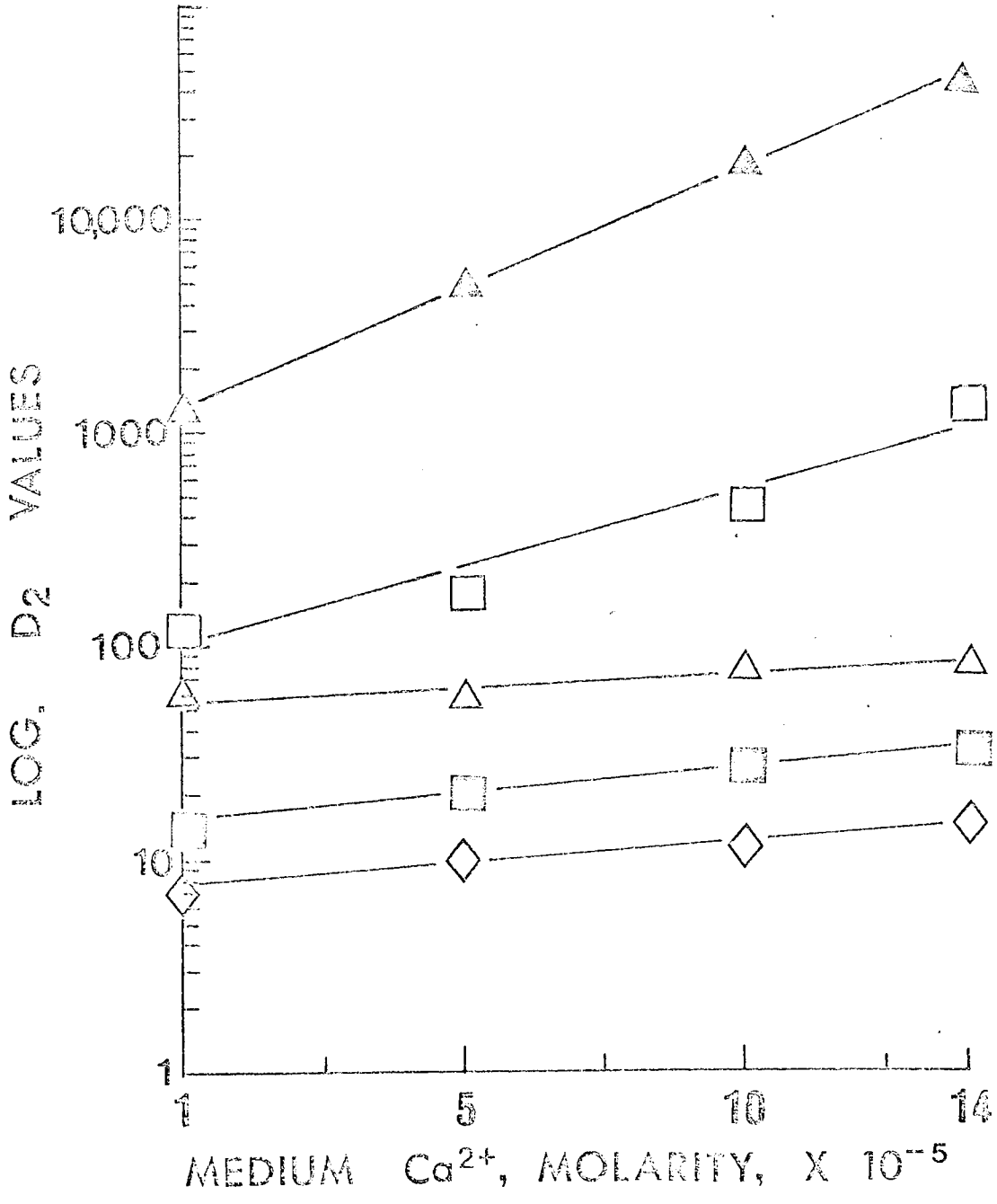
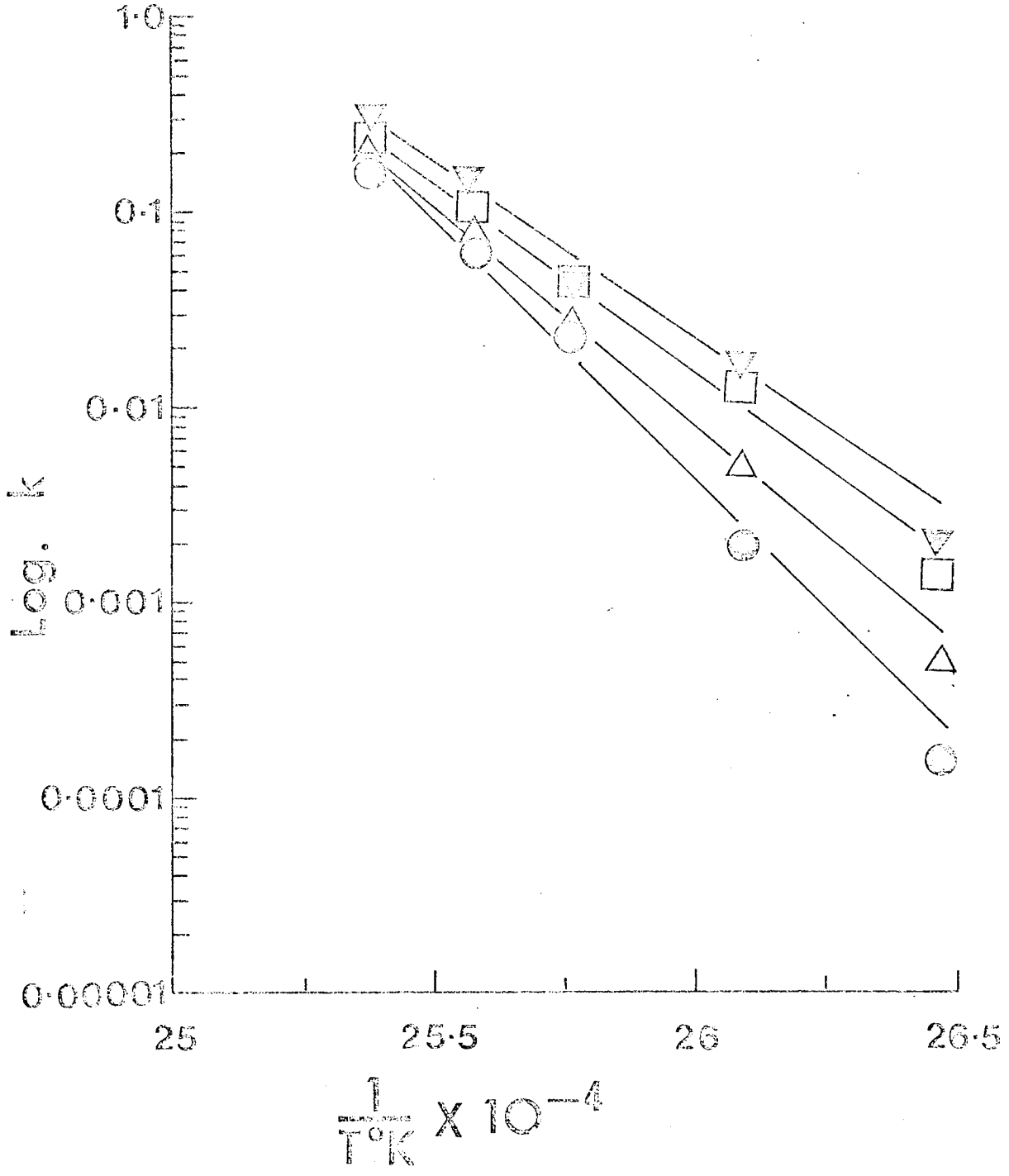

LOG. D2 VALUES
10,000
1000
100
10
1
1
5
10
14
MEDIUM Ca2+, MOLARITY, X 10−5

FIGURE 54

ARRHENIUS PLOTS FOR THE HEAT INACTIVATION OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED FROM NITROGEN DEPLETED CULTURES CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

MEDIUM CALCIUM (IN MOLAR)		ACTIVATION ENERGY (k cal./mol.)
▽—▽	1.0×10^{-5} M.	92.5
□—□	5.0×10^{-5} M.	111.0
△—△	1.0×10^{-4} M.	134.0
○—○	1.4×10^{-4} M.	148.0


1·0
0·1
0·01
0·001
0·0001
0·00001
25
25·5
26
26·5
$\frac{1}{T°K} \times 10^{-4}$

The entropy of activation (ΔS) was calculated from the relationship:

$$\Delta S = (Ea - RT - G)/T \qquad \text{(Moore, 1962).}$$

Since the value of RT tended to be small compared with experimental errors, this was often ignored (Stearn, 1949).

Table 30 shows the values of activation energy (Ea), free energy of activation (ΔG) and the entropy of activation (ΔS) calculated from the Arrhenius plots. The relationship between Ea, ΔS and the concentrations of calcium in the medium are shown in Figure 55. There is a positive correlation between the two parameters and medium calcium concentration.

Anderson and Friesen (1974) studied the thermal inactivation of B. stearothermophilus spores at various temperatures and reported the Arrhenius activation energy for spore inactivation to be 80 k cal./mol. at temperature above 105°C, the entropy of activation was quoted as 140-151 cal./mol./deg. They attributed this level of activation energy to be the rupture of many weak bonds probably hydrogen bonds associated with protein, leading to the collapse of the protein molecules with a consequent large increase in entropy. The activation energy and entropy of activation values obtained with N- spores (Table 30) were substantially higher than those reported by Anderson and Friesen (1974). If rupture of hydrogen bonds associated with protein is the mechanism for the thermal inactivation of spores, it is conceivable that the nature of these bonds are influenced by the

FIGURE 55

THE RELATIONSHIP BETWEEN ENERGIES OF ACTIVATION (□—□) OR ENTROPY OF ACTIVATION (●—●) AND THE CONCENTRATION OF SPORULATION CALCIUM OF SPORES OBTAINED FROM NITROGEN DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT)

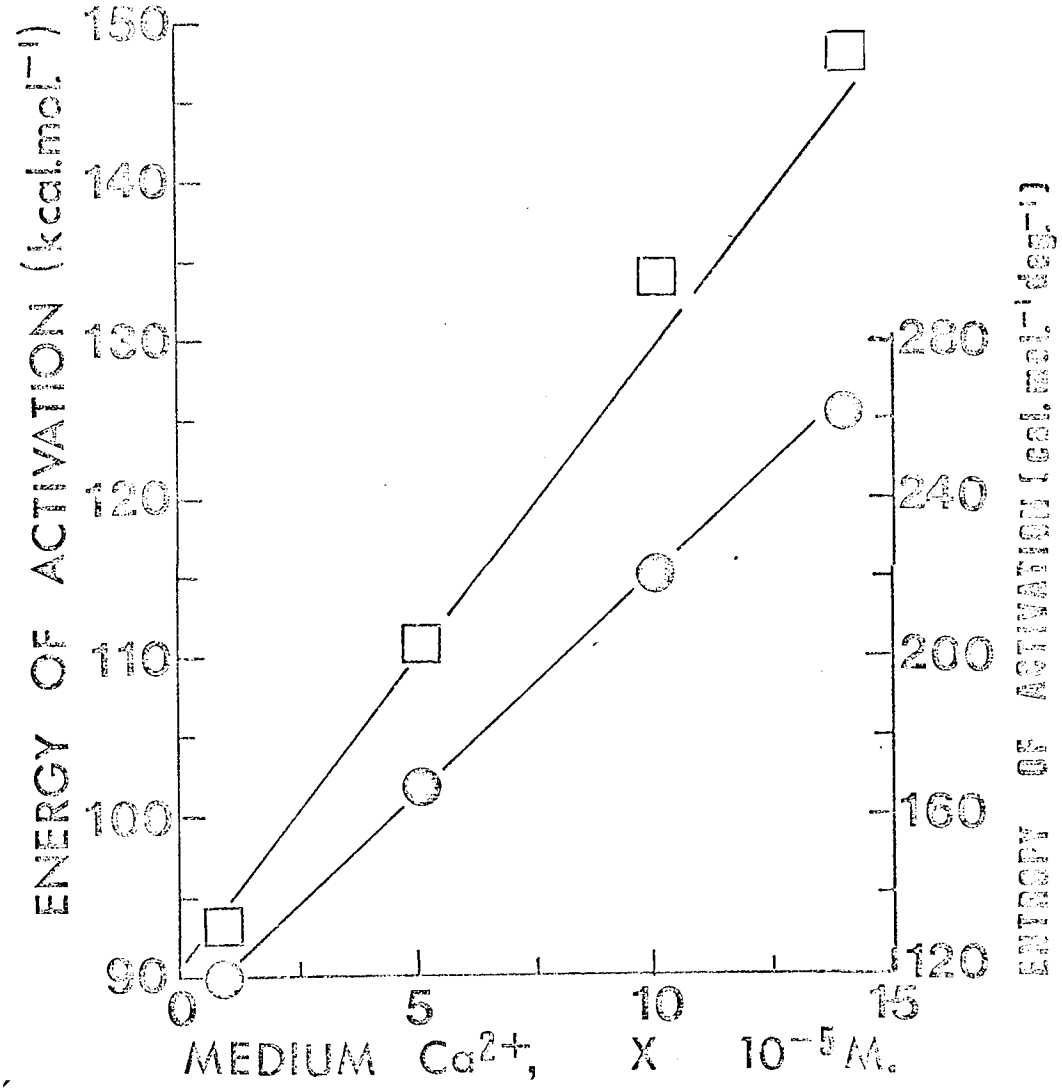

ENERGY OF ACTIVATION (kcal.mol.⁻¹)
ENTROPY OF ACTIVATION (cal.mol.⁻¹.deg.⁻¹)
150
140
130
120
110
100
90
280
240
200
160
120
0
5
10
15
MEDIUM Ca2+, X 10⁻⁵ M.

TABLE 30

THERMODYNAMIC FUNCTIONS OF HEAT INACTIVATION OF *B. STEAROTHERMOPHILUS* NCTC 10,003 (MUTANT) SPORES FROM NITROGEN DEPLETED CULTURES CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

SPORULATION CALCIUM CONC. (IN MOLAR)	HEATING TEMP. (°C)	ACTIVATION ENERGY (k cal./mol.)	FREE ENERGY OF ACTIVATION (k cal./mol.)	ENTROPY OF ACTIVATION (cal./mol./deg.)
1.4×10^{-4}	105	148	48.3	263
	110	148	46.7	264
	115	148	46.5	262
	118	148	46.2	260
	121	148	46.2	258
1.0×10^{-4}	105	134	48.5	226
	110	134	47.3	226
	115	134	46.6	225
	118	134	46.1	225
	121	134	45.7	224
5.0×10^{-5}	105	111	48.5	165
	110	111	46.5	168
	115	111	44.5	171
	118	111	45.9	166
	121	111	45.7	166
1.0×10^{-5}	105	92.5	47.4	119
	110	92.5	46.3	121
	115	92.5	46.3	119
	118	92.5	45.6	120
	121	92.5	45.4	120

nature of nutrient depletion. Since Arrhenius activation energy and entropy of activation were determined by the concentration of medium calcium (Figure 55), it is possible that the proteins postulated are in some ways stabilized by the spore calcium, which showed elevated levels in spores from media high in calcium (Table 29).

(b) The Effect Of Calcium Concentration in Sporulation Medium On the Germination of Spores Prepared From Nitrogen Depleted Cultures (N-).

The germination patterns of spores prepared from nitrogen depleted cultures containing different concentrations of calcium are shown in Figures 56-57. Germination occurred rapidly and extensively with N- spores obtained from media with different concentrations of calcium. This is in contrast to that observed with C- spores (Figure 41) where medium calcium higher than 1.0×10^{-5} M. resulted in the suppression of germination of such spores.

Arrhenius plots for the rate of germination of N- spores are shown in Figure 58. k, the rate constant was calculated from the linear part of the germination curves as percentage turned phase-dark per minute. The activation energy and other thermodynamic functions of germination were determined by regression (Table 31). The values obtained were comparable with those reported by Levinson and Hyatt (1970). They found activation energy of 20 k cal./mol. for the germination of B. megaterium spores and calculated values of 20-30 k cal./mol. for the germination of PA3679 spores from the data of Riemann (1963). Values of this magnitude were attributed

FIGURE 56

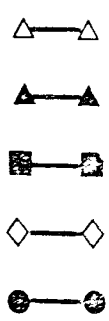
GERMINATION AT DIFFERENT TEMPERATURES OF SPORES OBTAINED FROM NITROGEN DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) CONTAINING GRADED CONCENTRATIONS OF CALCIUM

A) Spores obtained from medium containing 1.0×10^{-5} M. calcium

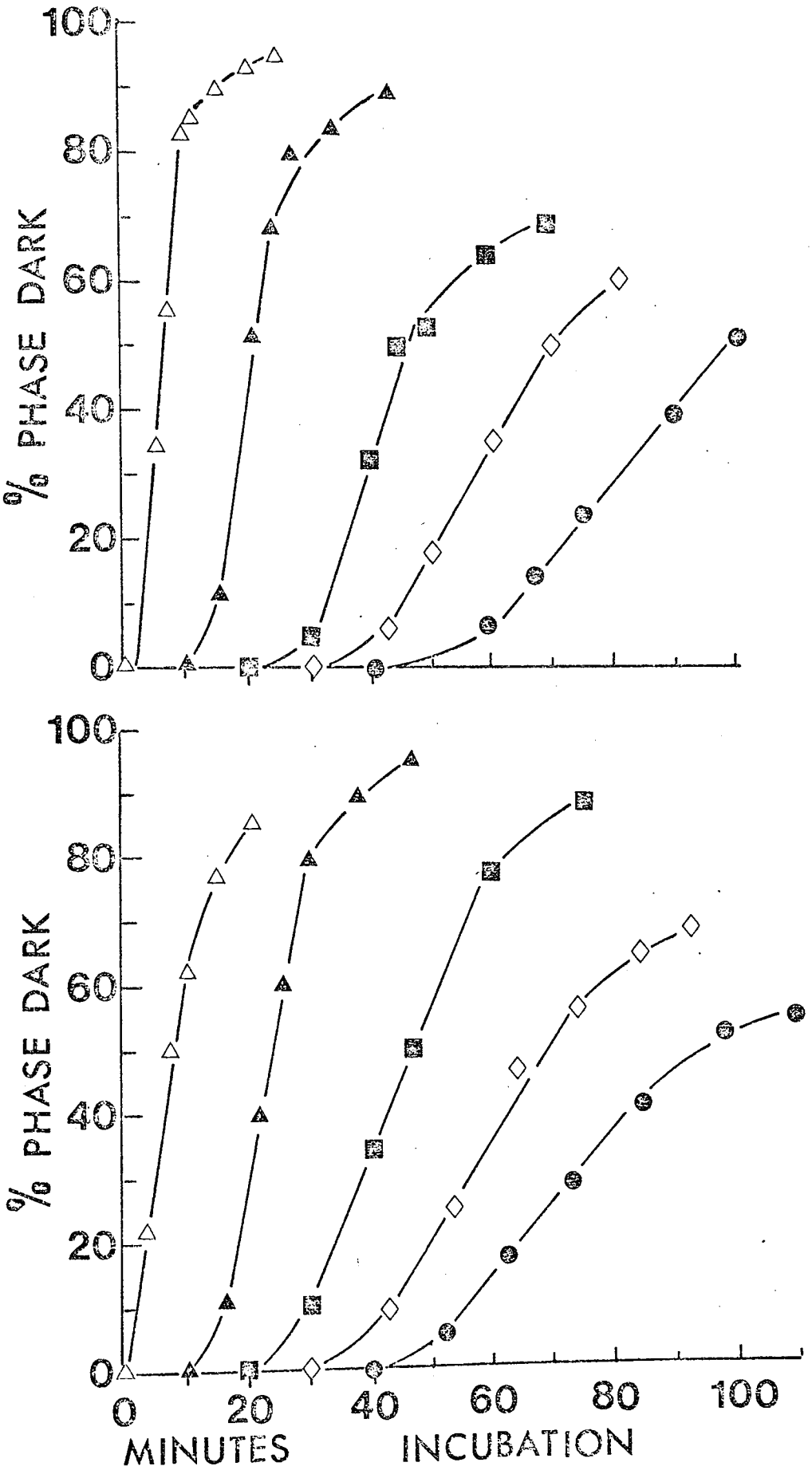
Germination temperature (°C)

B) Spores obtained from medium containing 5.0×10^{-5} M. calcium

Germination temperature (°C)



Note: Time scale displaced 10 mn. for each curve.



NOTE: TIME SCALE DISPLACED 10 MINUTES FOR EACH CURVE

FIGURE 57

GERMINATION AT DIFFERENT TEMPERATURES OF SPORES OBTAINED FROM NITROGEN DEPLETED CULTURES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) CONTAINING GRADED CONCENTRATIONS OF CALCIUM

C) Spores obtained from medium containing 1.0×10^{-4} M. calcium

Germination temperature ($^{\circ}$C)

△—△ 65°

▲—▲ 60°

■—■ 53°

◇—◇ 50°

●—● 48°

D) Spores obtained from medium containing 1.4×10^{-4} M. calcium

Germination temperature ($^{\circ}$C)

△—△ 66°

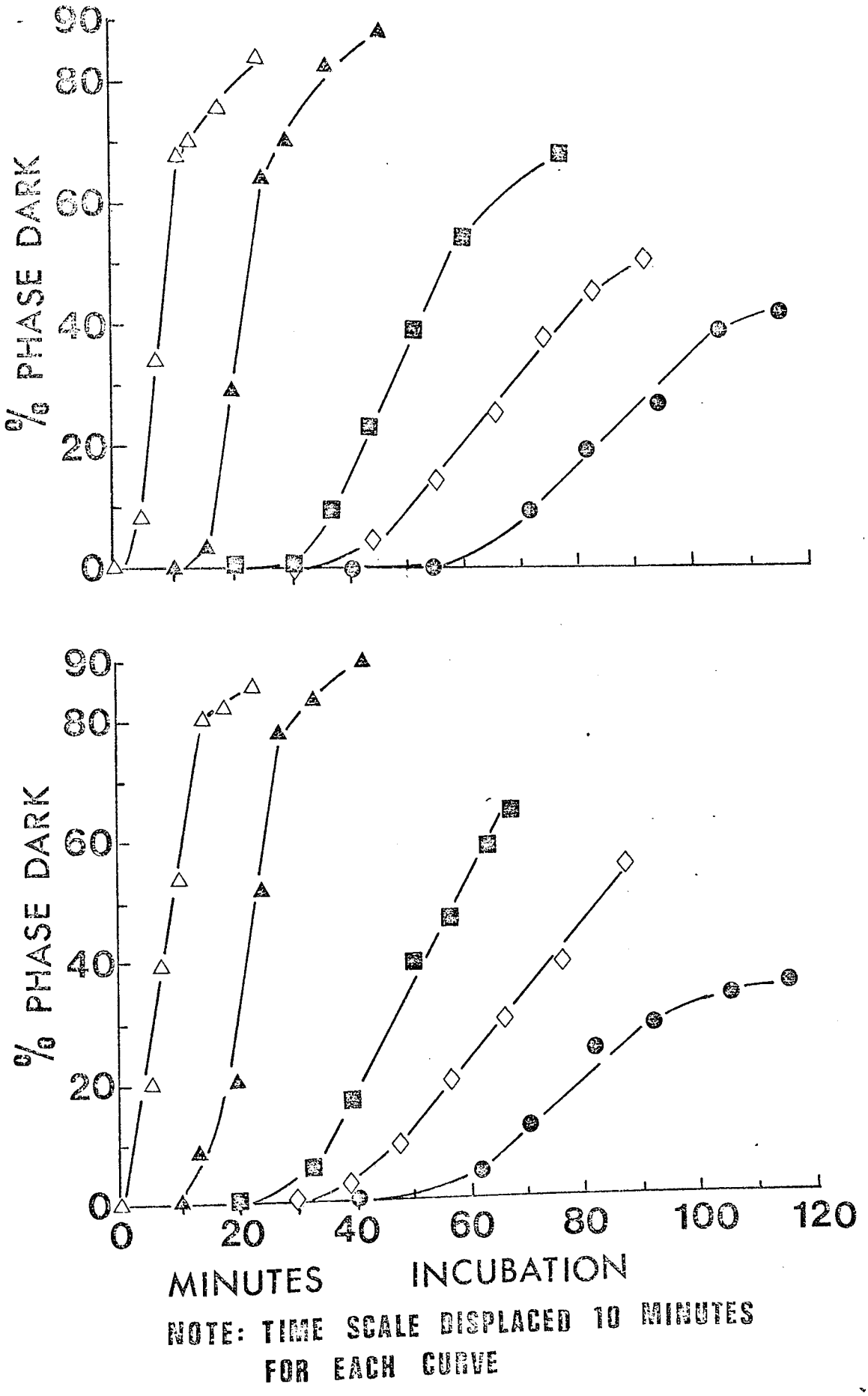
▲—▲ 60°

■—■ 53°

◇—◇ 50°

●—● 48°

Note: Time scale displaced 10 mn. for each curve.



NOTE: TIME SCALE DISPLACED 10 MINUTES FOR EACH CURVE

FIGURE 58

ARRHENIUS PLOTS OF GERMINATION RATES OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED FROM NITROGEN DEPLETED CULTURES CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

MEDIUM CALCIUM	ACTIVATION ENERGY (K cal./mol.)
○—○ 1.0×10^{-5} M.	25.8
□—□ 5.0×10^{-5} M.	23.3
▲—▲ 1.0×10^{-4} M.	31.0
▽—▽ 1.4×10^{-4} M.	27.5

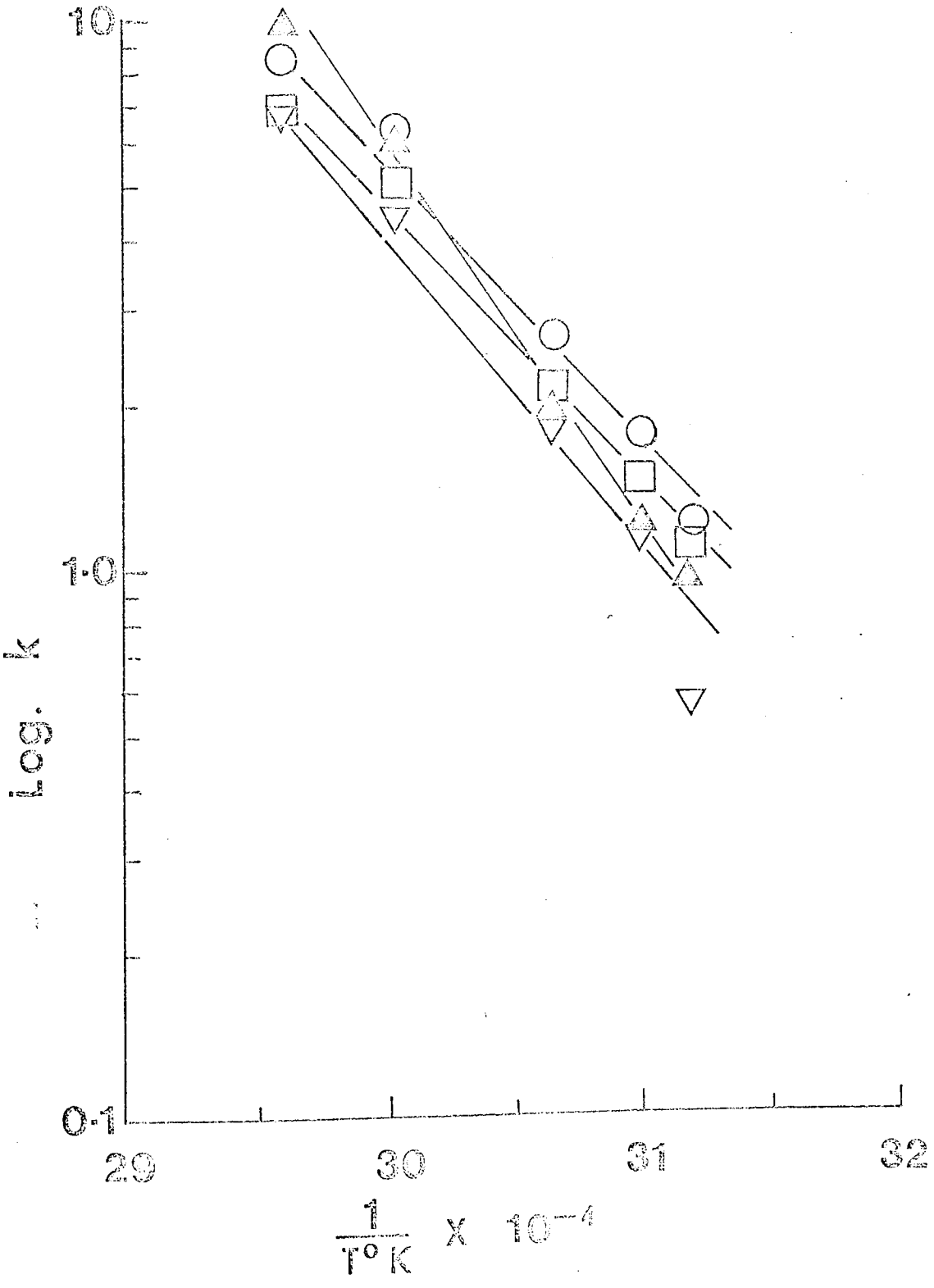

10
1·0
0·1
Log. K
29
30
31
32
1/T°K x 10⁻⁴

TABLE 31

THERMODYNAMIC FUNCTIONS OF GERMINATION RATE OF *B. STEAROTHERMOPHILUS* NCTC 10,003 (MUTANT) SPORES FROM NITROGEN DEPLETED CULTURES CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

SPORULATION CALCIUM CONC. (IN MOLAR)	GERMINATION TEMP. (°C)	ACTIVATION ENERGY (k cal./mol.)	FREE ENERGY OF ACTIVATION (k cal./mol.)	ENTROPY OF ACTIVATION (cal./mol./deg.)
1.4×10^{-4}	48	27.5	36.4	28
	50	27.5	36.3	27
	53	27.5	36.3	27
	60	27.5	36.5	27
	66	27.5	40.0	37
1.0×10^{-4}	48	31.0	36.2	16
	50	31.0	36.3	16
	53	31.0	36.3	16
	60	31.0	36.3	16
	65	31.0	36.5	16
5.0×10^{-5}	48	23.3	36.1	40
	50	23.3	36.1	40
	53	23.3	36.2	40
	60	23.3	36.4	39
	65	23.3	36.8	40
1.0×10^{-5}	48	25.8	36.0	32
	50	25.8	36.0	32
	53	25.8	36.1	32
	60	25.8	36.3	32
	65	25.8	36.6	32

to enzymatic reactions (Stearn, 1949).

(c) The Effect Of Calcium Concentration In Sporulation Medium On The Content Of Dipicolinic Acid, Calcium, Magnesium And Manganese Of Spores Prepared From Nitrogen Depleted Cultures (N-)

N- spores prepared from media containing different concentrations of calcium were analysed for D.P.A., calcium, magnesium and manganese concentrations as described in Section 2.K. to 2.M. The results are presented in Table 29. Lowering of medium calcium concentration resulted in a drop in the concentration of spore D.P.A. and calcium, with corresponding increase in the concentration of magnesium. The concentration of manganese showed very little change, spores from the lowest calcium medium appeared to have slightly elevated concentration of manganese. The molar ratio of calcium to D.P.A. is close to unity, but Mg/Ca molar ratio increased significantly with the decrease of calcium concentration in spores, this was accompanied by the fall of spore resistance to heat.

(d) The Effect Of Calcium Concentration In Sporulation Medium On The Release Of Dipicolinic Acid From Spores Obtained From Nitrogen Depleted Cultures (N-)

The effect of different concentrations of medium calcium on the rate of D.P.A. release from N- spores were determined as described in Section 2.P. The patterns of dipicolinic acid release are shown in Figures 59 - 60.

The rate of release was determined by the temperature of

FIGURE 52

THE RELEASE OF DIPICOLINIC ACID FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED IN NITROGEN DEPLETED CULTURES CONTAINING GRADED CONCENTRATIONS OF CALCIUM

A) Spores obtained from medium containing 1.0×10^{-5} M. calcium

Release temperature (°C)

△—△ 98°

◎—◎ 95°

○—○ 91°

◇—◇ 86°

▽—▽ 80°

B) Spores obtained from medium containing 5.0×10^{-5} M. calcium

Release temperature (°C)

△—△ 95°

◎—◎ 91°

○—○ 88°

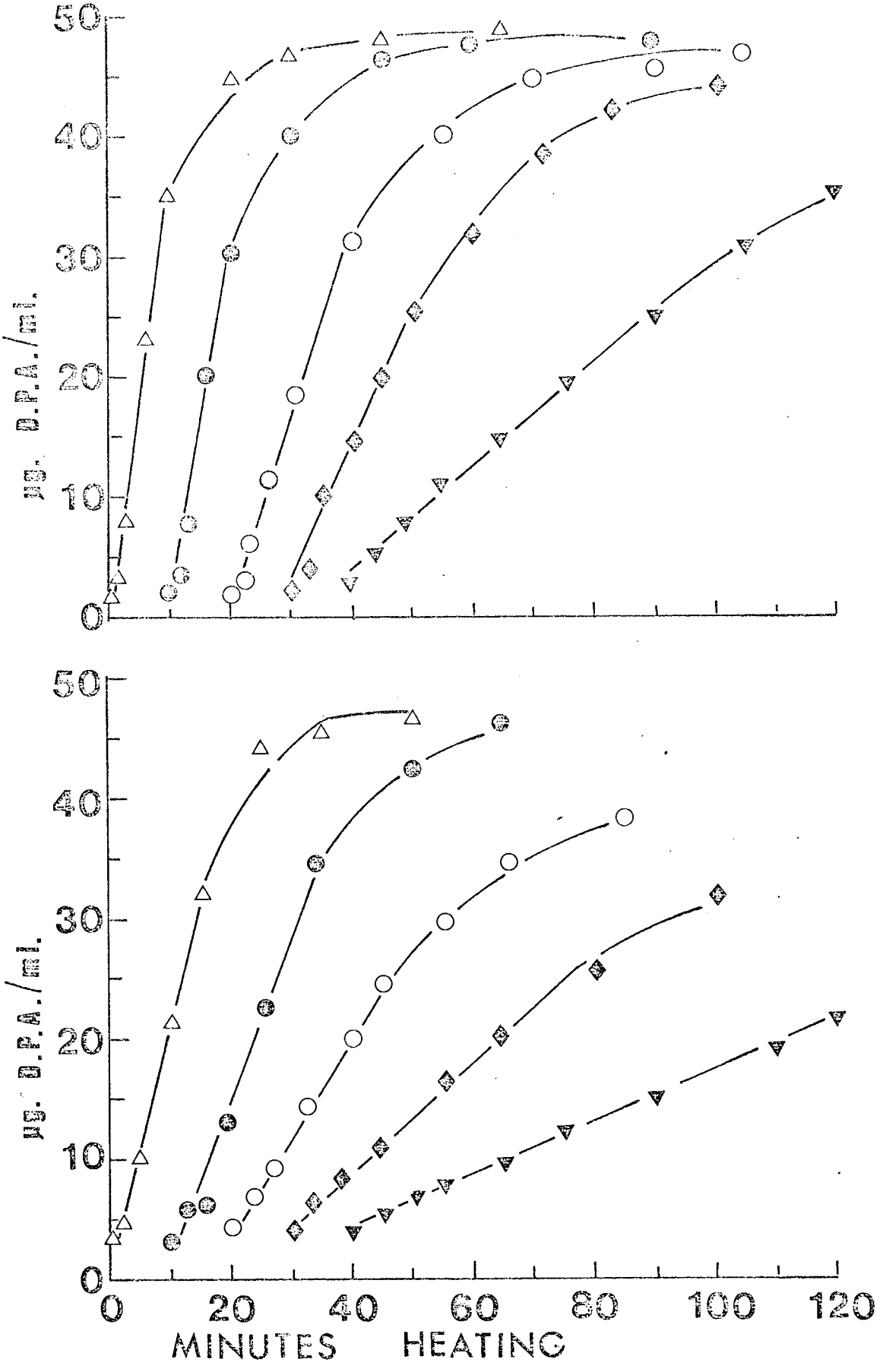
◇—◇ 84°

▽—▽ 80°

Note: 1. Spores were suspended in citrate buffer, pH 4 in each case.

2. The spore concentration used was normalized to a fixed concentration of 2.82×10^{8} spores/ml. in each case.

3. Time scale displaced 10 minutes for each curve.



NOTE: TIME SCALE DISPLACD 10 MINUTES FOR EACH CURVE

FIGURE 60

THE RELEASE OF DIPICOLINIC ACID FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED IN NITROGEN DEPLETED CULTURES CONTAINING GRADED CONCENTRATIONS OF CALCIUM

C) Spores obtained from medium containing 1.0×10^{-4} M. calcium

Release temperature ($^{\circ}$C)

△—△ 100°

●—● 95°

□—□ 91°

○—○ 88°

▽—▽ 80°

D) Spores obtained from medium containing 1.4×10^{-4} M. calcium.

Release temperature ($^{\circ}$C)

△—△ 95°

●—● 91°

■—■ 88°

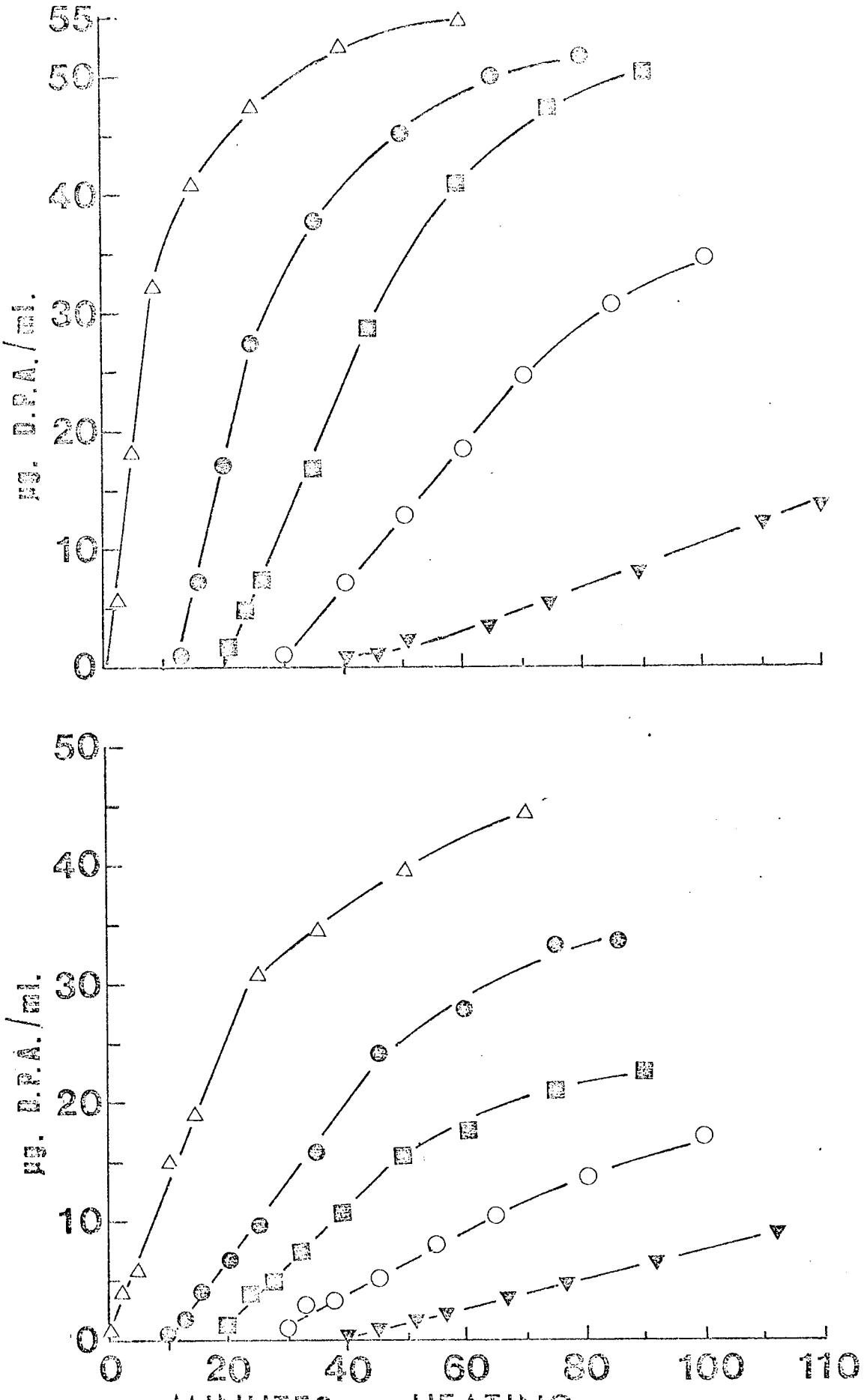
○—○ 84°

▽—▽ 80°

Note: 1. Spores were suspended in citrate buffer, pH 4 in each case.

2. The spore concentration used was normalized to a fixed concentration of 2.82×10^{8} spores/ml. in each case.

3. Time scale displaced 10 minutes for each curve.



NOTE: TIME SCALE DISPLACED 10 MINUTES FOR EACH CURVE

FIGURE 61

ARRHENIUS PLOTS OF D.P.A. RELEASE FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED FROM NITROGEN DEPLETED CULTURES CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

SPORULATION CALCIUM	ACTIVATION ENERGY (k cal./mol.)
△—△ 1.0×10^{-5} M.	31.2
◎—◎ 5.0×10^{-5} M.	40.5
□—□ 1.0×10^{-4} M.	41.2
▲—▲ 1.4×10^{-4} M.	39.3

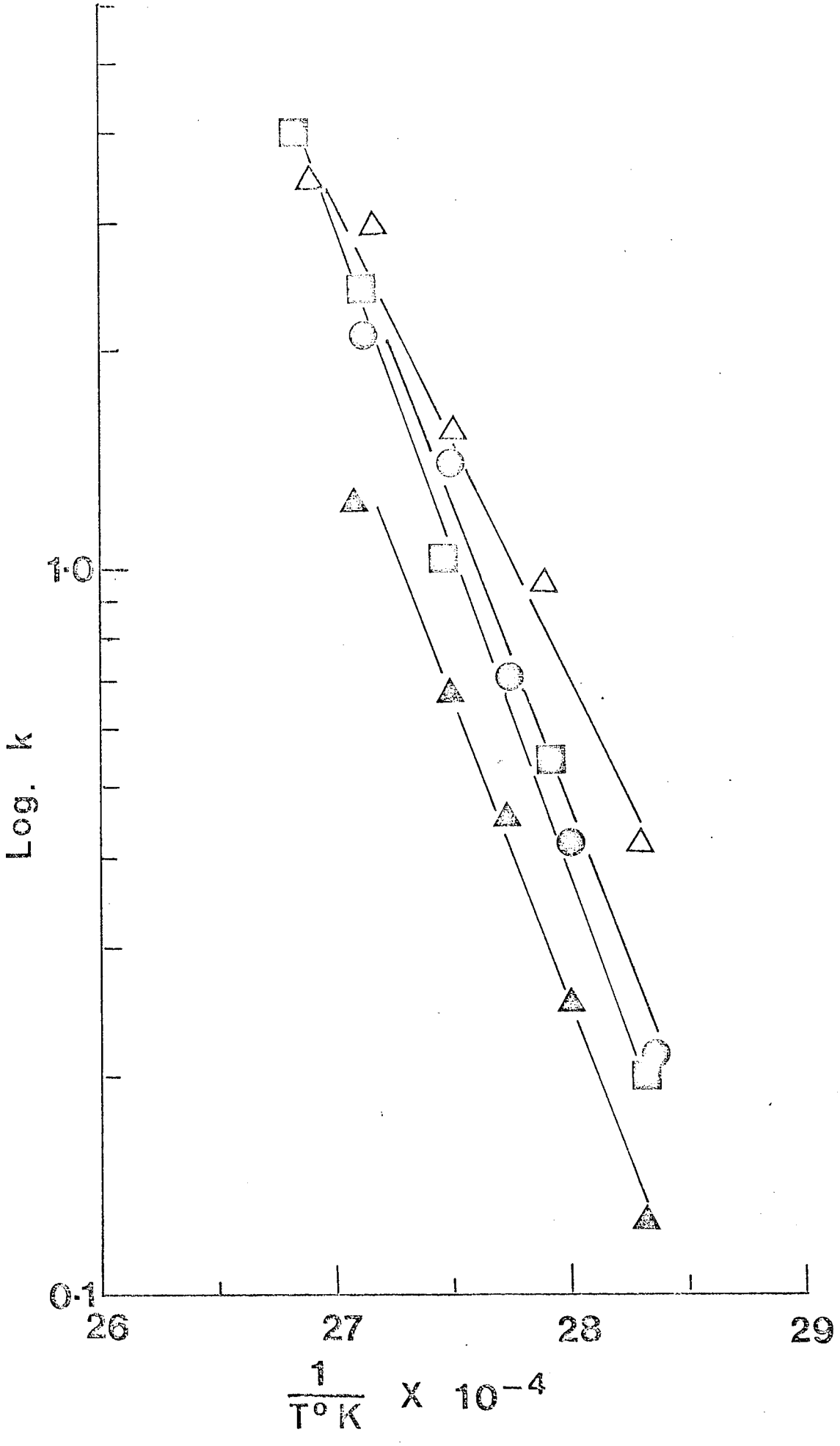

Log. k
1·0
0·1
26
27
28
29
1/T°K X 10⁻⁴

TABLE 32

THERMODYNAMIC FUNCTIONS FOR THE RELEASE OF DIPICOLINIC ACID FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED IN NITROGEN DEPLETED CULTURES CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

SPORULATION CALCIUM CONC. (IN MOLAR)	RELEASE TEMP. (°C)	ACTIVATION ENERGY (k cal./mol.)	FREE ENERGY OF ACTIVATION (k cal./mol.)	ENTROPY OF ACTIVATION (cal./mol./deg.)
1.4×10^{-4}	80	39.5	41.3	5.1
	84	39.5	41.3	5.0
	88	39.5	41.3	5.0
	91	39.5	41.4	5.2
	95	39.5	41.4	5.2
1.0×10^{-4}	80	34.3	41.0	19.0
	86	34.3	40.9	18.0
	91	34.3	41.0	18.0
	95	34.3	40.9	18.0
	100	34.3	41.0	18.0
5.0×10^{-5}	80	39.8	40.9	3.0
	84	39.8	40.9	3.0
	88	39.8	41.0	3.0
	91	39.8	40.8	3.0
	95	39.8	41.0	3.0
1.0×10^{-5}	80	33.6	40.4	20.0
	86	33.6	40.5	20.0
	91	33.6	40.8	20.0
	95	33.6	40.7	20.0
	98	33.6	41.0	20.0

Note: Spores were suspended in citrate buffer, pH 4 in each case

heating, thus confirming the observation of Brown and Melling (1973) that the release of D.P.A. and calcium from spores is a function of time, temperature and pH. Attempts to determine the release of D.P.A. at pH lower than 4 was unsuccessful, the spores lyzed at temperature greater than 90°C at pH < 4.

Arrhenius plots for D.P.A. release of N- spores prepared from media containing different concentrations of calcium are shown in Figure 61. k, the rate constant was determined from the linear part of the release curves as µg. D.P.A./ml./mn. The slope of the Arrhenius plots was determined by regression analysis and the thermodynamic data calculated (Table 32). The activation energy for D.P.A. release was in the range of 33.6 to 39.8 k cal./mol., the entropy of activation ranges from 3 to 20 cal./mol./deg. The activation energy was not affected by the concentration of medium calcium. Brown and Melling (1973) found the apparent activation energy for D.P.A. release to be 46 k cal./mol. for Bacillus stearothermophilus NCIB 8919 spores prepared from washing of agar surfaces.

(e) The Effect of Calcium Concentration in Sporulation Medium On The Release of Calcium From Spores Obtained From Nitrogen Depleted Cultures (N-).

The effects of different concentrations of medium calcium on the rate of calcium release from N- spores were determined as described in Section 2.P. The patterns of calcium release are shown in Figures 62-63. Arrhenius plots of calcium release from N- spores prepared in media containing different concentrations

FIGURE 62

THE RELEASE OF CALCIUM FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED IN NITROGEN DEPLETED CULTURES CONTAINING GRADED CONCENTRATIONS OF CALCIUM

A) Spores obtained from medium containing 1.0×10^{-5} M. calcium

Release temperature (°C)

△—△ 98°

●—● 95°

◇—◇ 91°

◈—◈ 86°

○—○ 80°

B) Spores obtained from medium containing 5.0×10^{-5} M. calcium

Release temperature (°C)

△—△ 95°

●—● 91°

◇—◇ 88°

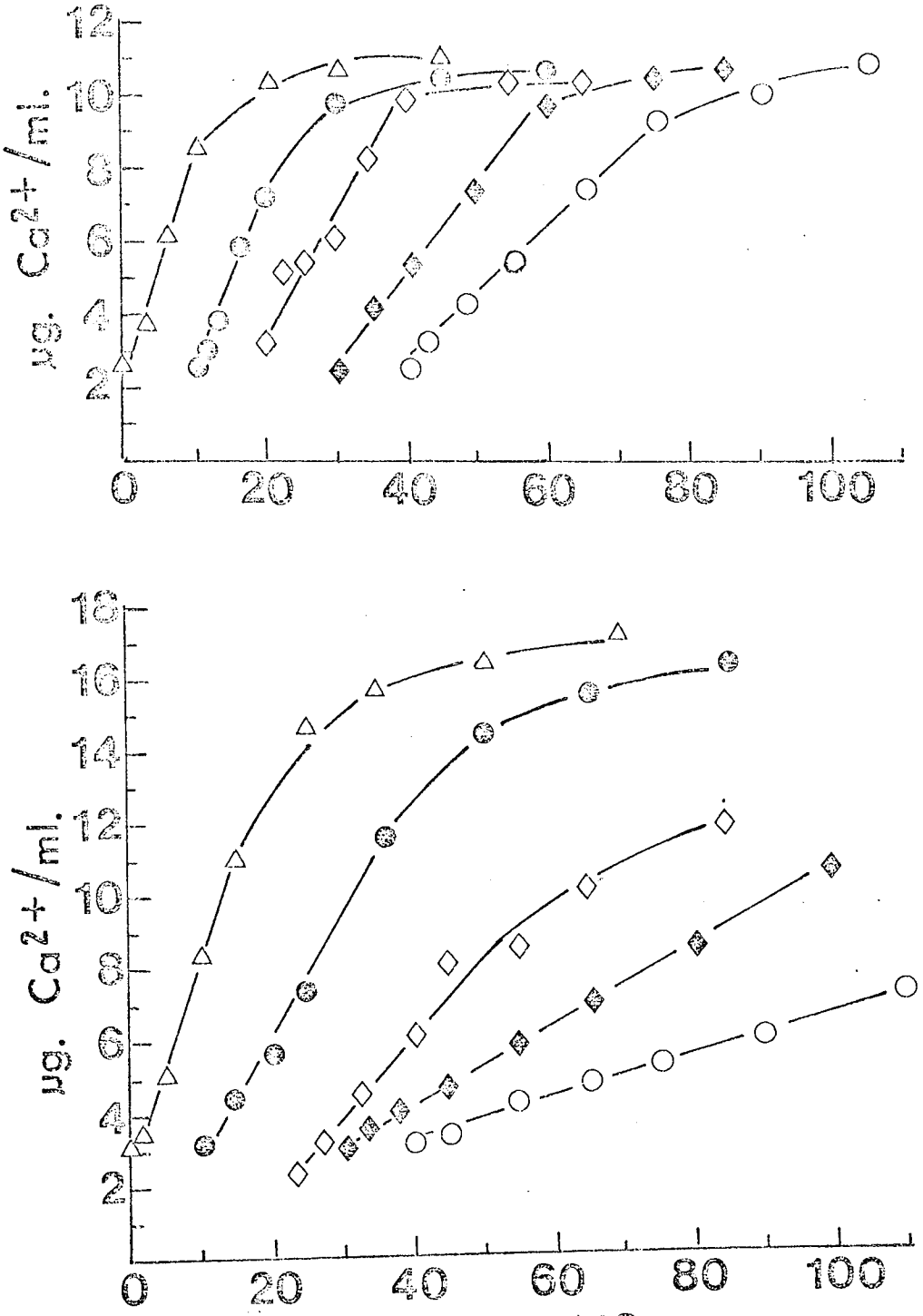
◈—◈ 84°

○—○ 80°

Note: 1. Spores were suspended in citrate buffer, pH 4 in each case.

2. All readings were corrected by assuming that a fixed concentration of spores equal to 2.82×10^{8} spores/ml. were used in each case.

3. Time scale displaced 10 mn. for each curve.



NOTE: TIME SCALE DISPLACED 10 MINUTES FOR EACH CURVE

FIGURE 63

THE RELEASE OF CALCIUM FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED IN NITROGEN DEPLETED CULTURES CONTAINING GRADED CONCENTRATIONS OF CALCIUM

C) Spores obtained from medium containing 1.0×10^{-4} M. calcium

Release temperature (^{o}C)

△—△ 100°

●—● 95°

○—○ 91°

▲—▲ 86°

■—■ 80°

D) Spores obtained from medium containing 1.4×10^{-4} M. calcium

Release temperature (^{o}C)

△—△ 95°

●—● 91°

○—○ 88°

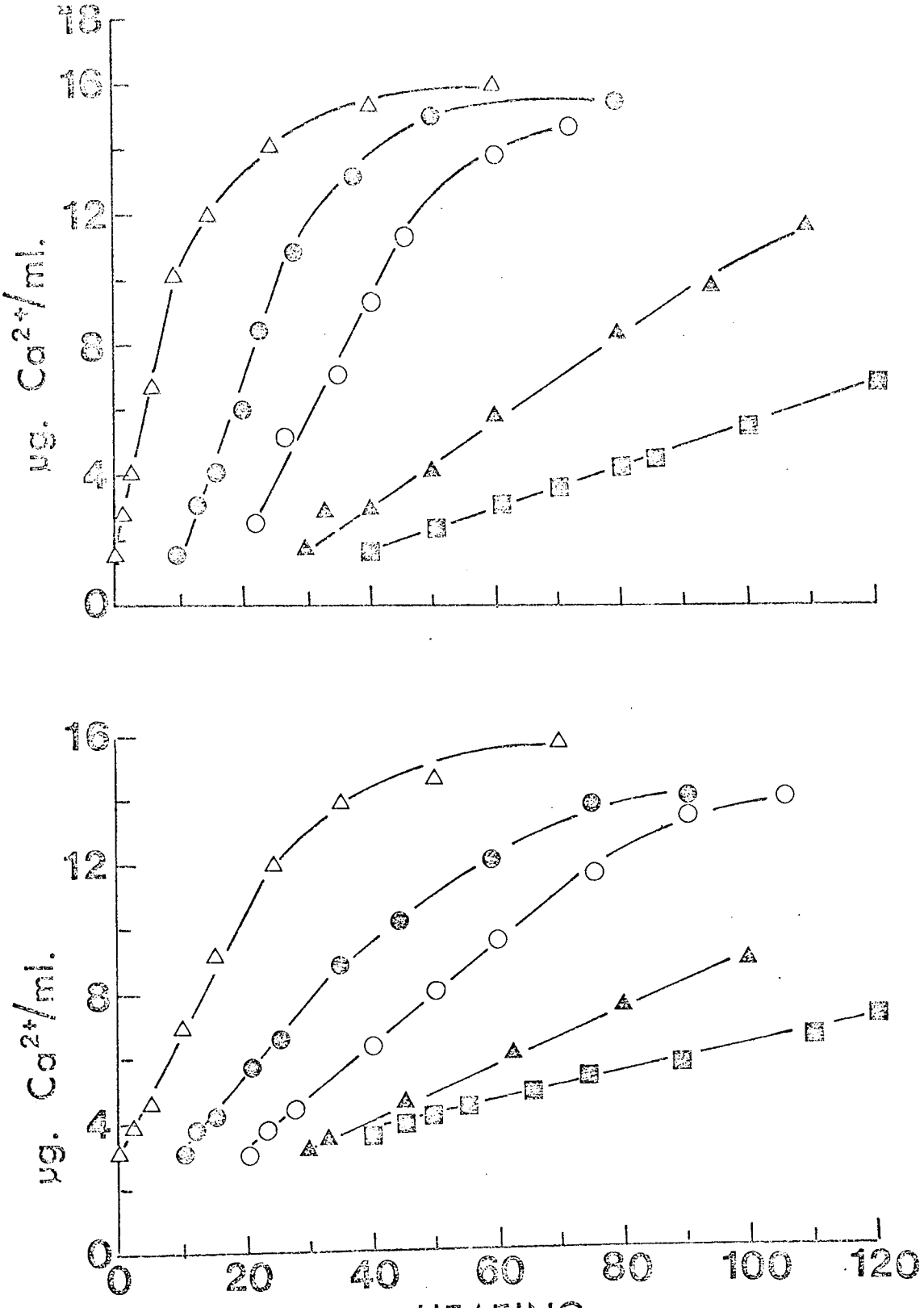
▲—▲ 84°

■—■ 80°

Note: 1. Spores were suspended in citrate buffer, pH 4 in each case.

2. The spore concentration used was normalized to a fixed concentration of 2.82×10^{8} spores/ml. in each case.

3. Time scale displaced 10 minutes for each curve.



NOTE: TIME SCALE DISPLACED
10 MINUTES FOR EACH CURVE

FIGURE 64

ARRHENIUS PLOTS OF CALCIUM RELEASE FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED FROM NITROGEN DEPLETED CULTURES CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

SPORULATION CALCIUM (IN MOLAR)	ACTIVATION ENERGY (k cal./mol.)
△—△ 1.0×10^{-5}	16.4
◈—◈ 5.0×10^{-5}	41.2
□—□ 1.0×10^{-4}	36.6
⊕—⊕ 1.4×10^{-4}	37.5

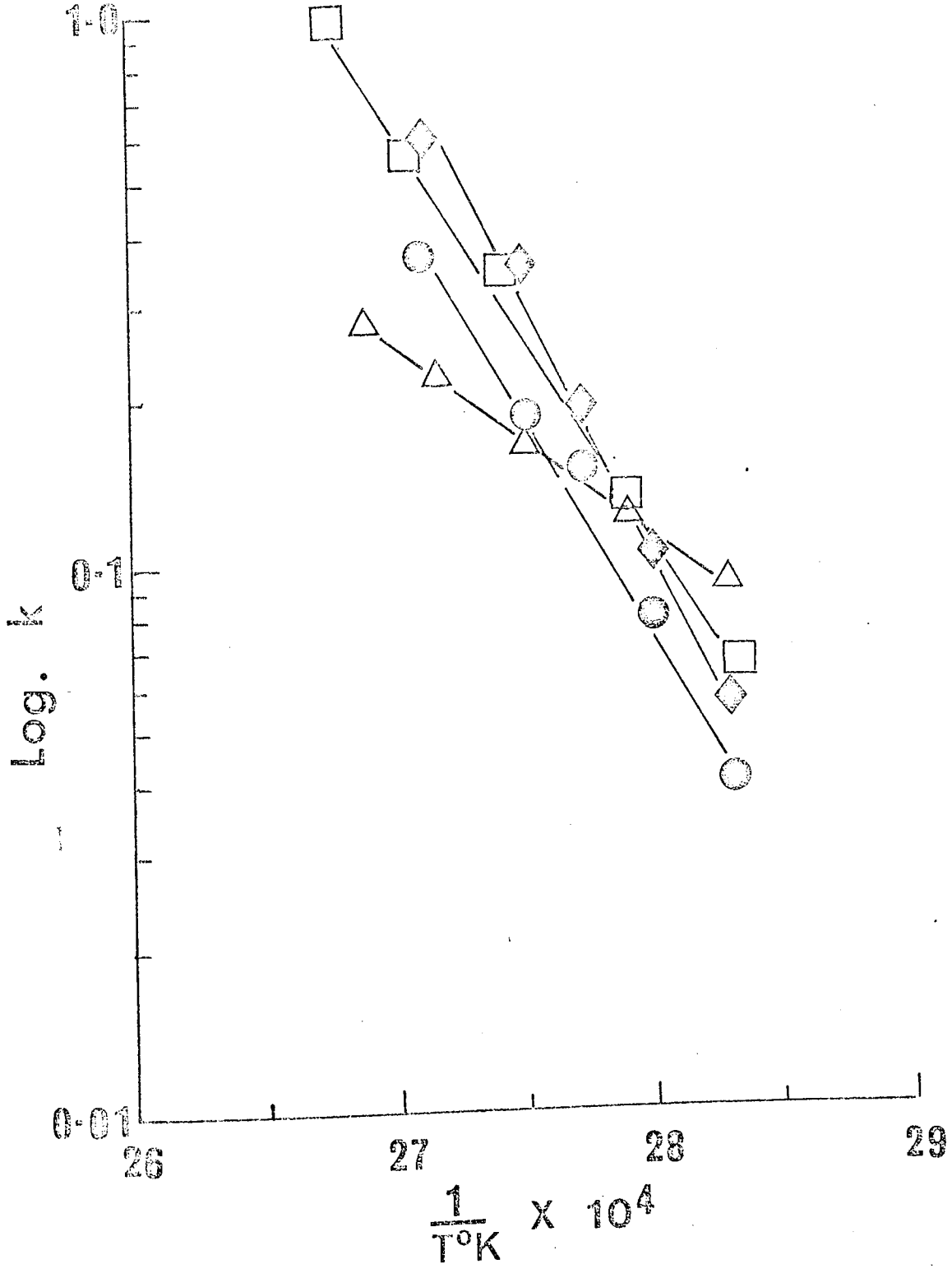

1·0
0·1
0·01
Log. k
26
27
28
29
1/T°K x 10⁴

TABLE 33

THERMODYNAMIC FUNCTIONS FOR THE RELEASE OF CALCIUM FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED IN NITROGEN DEPLETED CULTURES CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

SPORULATION CALCIUM CONC. (IN MOLAR)	RELEASE TEMP. (°C)	ACTIVATION ENERGY (k cal./mol.)	FREE ENERGY OF ACTIVATION (k cal./mol.)	ENTROPY OF ACTIVATION (cal./mol./deg.)
1.4×10^{-4}	80	34.3	40.5	18
	84	34.3	42.0	22
	88	34.3	42.1	22
	91	34.3	42.3	22
	95	34.3	42.2	22
1.0×10^{-4}	80	28.2	41.5	38
	86	28.2	43.4	42
	91	28.2	42.4	39
	95	28.2	42.6	39
	98	28.2	42.8	39
5.0×10^{-5}	80	37.0	41.8	14
	84	37.0	41.8	13
	88	37.0	41.9	14
	91	37.0	41.8	13
	95	37.0	41.9	13
1.0×10^{-5}	80	17.1	41.4	69
	86	17.1	41.9	69
	91	17.1	41.8	68
	95	17.1	42.1	68
	100	17.1	42.1	67

Note: Spores were suspended in citrate buffer, pH 4 in each case.

of calcium are shown in Figure 64. k, the rate constant was determined from the linear part of the release curves as μg Ca^{2+} released/ml./mn. The slope of the Arrhenius plots was determined by regression analysis and the thermodynamic data calculated (Table 33). The activation energy (Ea) for Ca^{2+} release was low with spores from 1.0×10^{-5} M. Ca^{2+} medium (17.1 k cal./mol.) and high (28.2 to 37.0 k cal./mol.) for spores from higher calcium medium. The reason for the low Ea value of spores from 1.0×10^{-5} M. Ca^{2+} medium is not known. The Ea values for calcium and D.P.A. release were close but probably not the same.

(f) <u>The Effect of Calcium Concentration in Sporulation Medium on The Release of Magnesium And Manganese From Spores Obtained From Nitrogen Depleted Cultures (N-)</u>.

The spore concentration adequate for the determination of D.P.A. and calcium release was unfortunately not suitable for the study of magnesium and manganese release. Preliminary studies showed that the amount of magnesium present in the medium at time zero was high while the amount of magnesium released following heating was smaller than the background magnesium and so could not be measured accurately at low spore concentrations. At the spore concentration used for studying D.P.A. and calcium release, the concentration of manganese released after maximal heating was measurable, but the concentration below the maximal heating time was too low to be detected by the instrument used.

To overcome the problem, spore concentrations higher than those used for studying D.P.A. and calcium release were used.

A fixed concentration of spores (~ 8 x 10^8 spores/ml.) in citrate buffer, pH 4 was heated for exactly 10 minutes at temperatures ranging from 80--95°C, the amount of magnesium and manganese released were determined as described in Section 2.P. The controls consisted of equal concentration of spore suspension in citrate buffer, pH4, but kept at room temperature. Preliminary studies had shown that up to 10 mn. heating, there was a linear relationship between the amount of cation release and the duration of heating. This relationship was obeyed even at the maximum heating temperature used in the present study.

The rate of magnesium and manganese release per minute was shown in Table 34, after correcting for the readings in the controls and normalizing the spore concentration used to 2.82 x 10^8 spores/ml. (the spore concentration used in the study of release of other spore components). k, the first order rate constant is equal to µg. cation released/ml./mn. Arrhenius plots for magnesium and manganese release are shown in Figures 65 and 66. Energies of activation were calculated to be 20.5 to 30.4 and 25.5 to 34.6 k cal./mol. for the release of magnesium and manganese respectively. The entropy of activation was 33 to 63 cal./mol./deg. for magnesium release and 37 to 51 cal./mol./deg. for manganese release. The free energy of activation was 41.9 to 44.4 k cal./mol. for magnesium release and 42.1 to 44.4 k cal./mol. for manganese release (Tables 35 and 36). Values of this magnitude are within the range expected for enzymatic reactions (Stearn, 1949).

TABLE 34

THE RELEASE OF MAGNESIUM AND MANGANESE FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED IN NITROGEN DEPLETED CULTURES CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

SPORULATION CALCIUM CONC. (IN MOLAR)	RELEASE TEMP. (°C)	RELEASE OF: MAGNESIUM ($\times 10^{-2}$ µg./*ml./mn.)	RELEASE OF: MANGANESE ($\times 10^{-2}$ µg./*ml./mn.)
1.4×10^{-4}	80	2.00	0.38
	84	2.14	0.43
	88	2.50	0.75
	91	4.28	1.25
	95	6.66	2.10
1.0×10^{-4}	80	1.90	0.70
	86	2.50	1.25
	91	4.50	2.10
	95	6.00	3.00
	100	8.60	5.00
5.0×10^{-5}	80	3.20	0.57
	84	4.00	0.90
	88	6.00	1.55
	91	8.18	2.10
	95	15.00	4.50
1.0×10^{-5}	80	4.90	1.10
	86	11.50	1.90
	91	20.00	3.50
	95	32.00	5.80
	98	40.00	7.80

Note: Spores were suspended in citrate buffer, pH 4 in each case.

* Spore concentration normalized to 2.82×10^{8} spores/ml. in each case.

FIGURE 65

ARRHENIUS PLOTS OF MAGNESIUM RELEASE FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED FROM NITROGEN DEPLETED CULTURES CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

SPORULATION CALCIUM (IN MOLAR)	ACTIVATION ENERGY (k cal./mol.)
▣—▣ 1.0×10^{-5}	30.4
◇—◇ 5.0×10^{-5}	26.3
▽—▽ 1.0×10^{-4}	20.5
△—△ 1.4×10^{-4}	21.2

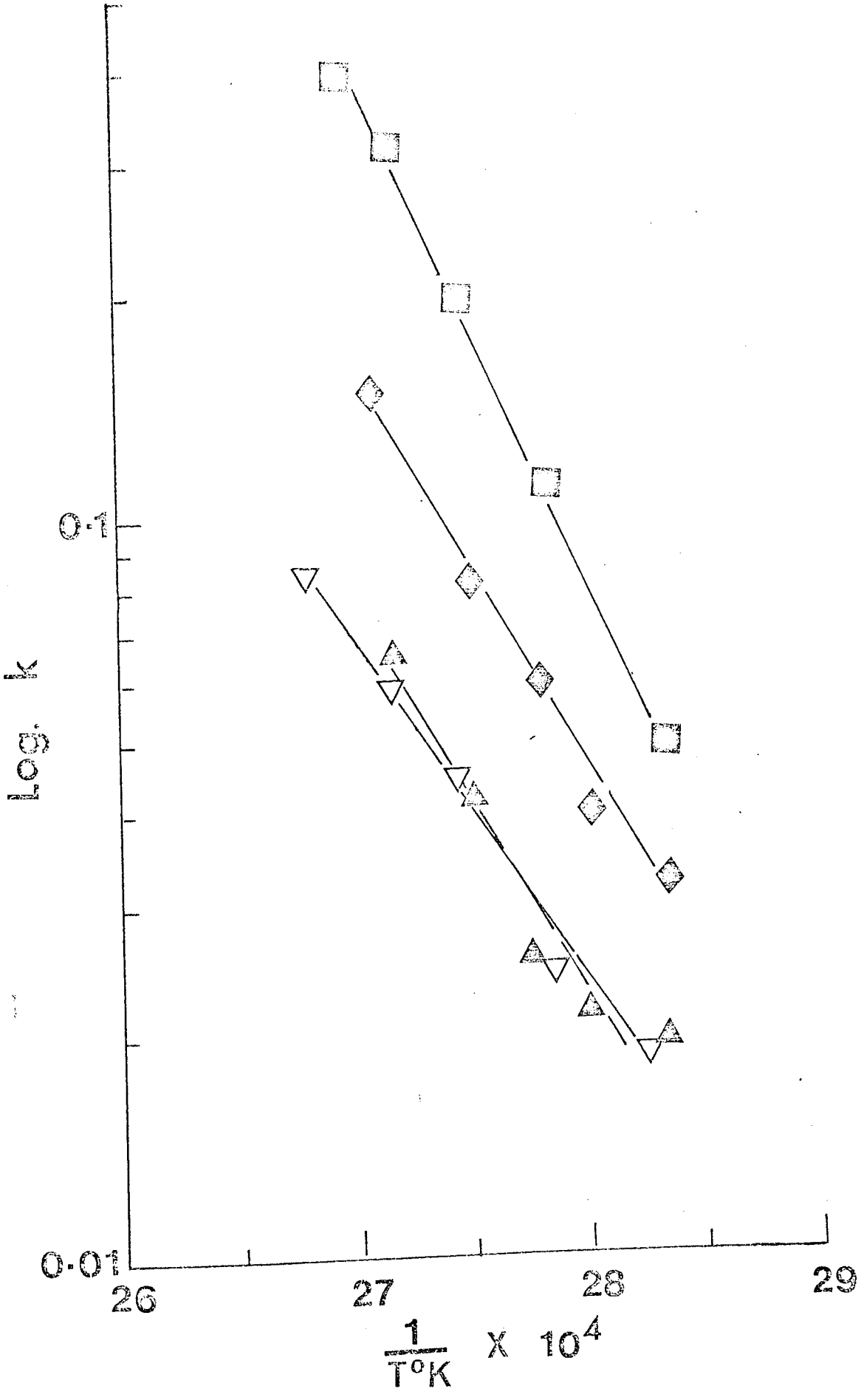

0·1
0·01
Log K
26
27
28
29
1/T°K X 10⁴

FIGURE 66

ARRHENIUS PLOTS OF MANGANESE RELEASE FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED FROM NITROGEN DEPLETED CULTURES CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

SPORULATION CALCIUM (IN MOLAR)	ACTIVATION ENERGY (k cal./mol.)
□—□ 1.0×10^{-5}	28.9
△—△ 5.0×10^{-5}	34.6
○—○ 1.0×10^{-4}	25.5
▽—▽ 1.4×10^{-4}	30.8

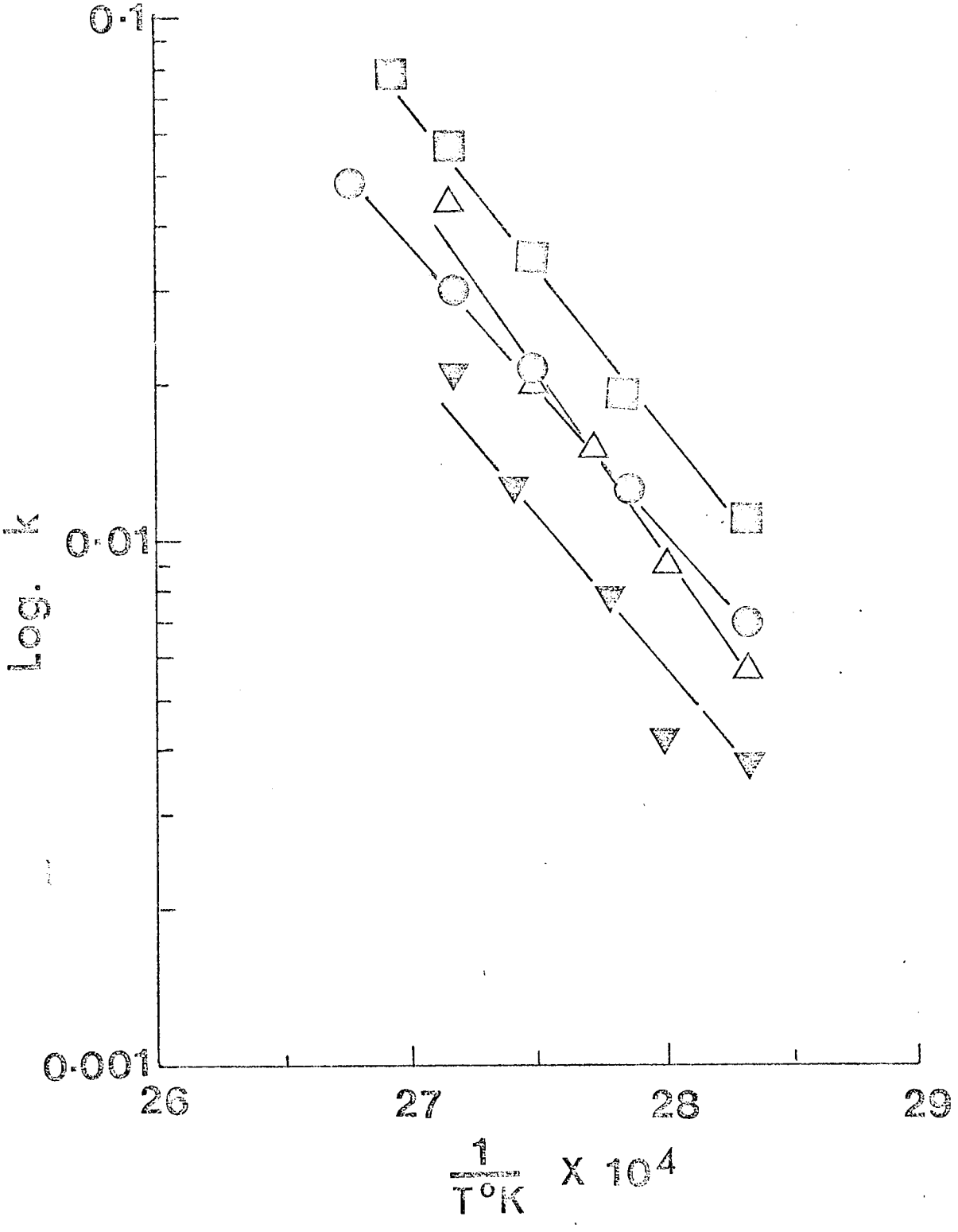

0·1
0·01
0·001
Log. χ
26
27
28
29
1/T°K X 10⁴

TABLE 35

THERMODYNAMIC FUNCTIONS FOR THE RELEASE OF MAGNESIUM FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED IN NITROGEN DEPLETED CULTURES CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

SPORULATION CALCIUM CONC. (IN MOLAR)	RELEASE TEMP. (°C)	ACTIVATION ENERGY (k cal./mol.)	FREE ENERGY OF ACTIVATION (k cal./mol.)	ENTROPY OF ACTIVATION (cal./mol./deg.)
1.4×10^{-4}	80	21.2	43.0	62
	84	21.2	43.0	61
	88	21.2	43.4	61
	91	21.2	43.4	61
	95	21.2	44.4	63
1.0×10^{-4}	80	20.5	42.6	63
	86	20.5	43.1	63
	91	20.5	43.3	63
	95	20.5	43.6	63
	100	20.5	43.9	63
5.0×10^{-5}	80	26.3	42.2	45
	84	26.3	42.5	45
	88	26.3	42.7	45
	91	26.3	42.9	46
	95	26.3	42.9	45
1.0×10^{-5}	80	30.4	41.9	33
	86	30.4	42.0	32
	91	30.4	42.2	32
	95	30.4	42.4	33
	98	30.4	42.6	33

Note: Spores were suspended in citrate buffer, pH 4 in each case.

TABLE 36

THERMODYNAMIC FUNCTIONS FOR THE RELEASE OF MANGANESE FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED IN NITROGEN DEPLETED CULTURES CONTAINING DIFFERENT CONCENTRATIONS OF CALCIUM

SPORULATION CALCIUM CONC. (IN MOLAR)	RELEASE TEMP. (°C)	ACTIVATION ENERGY (k cal./mol.)	FREE ENERGY OF ACTIVATION (k cal./mol.)	ENTROPY OF ACTIVATION (cal./mol./deg.)
1.4×10^{-4}	80	30.8	43.7	37
	84	30.8	44.1	37
	88	30.8	44.2	37
	91	30.8	44.2	37
	95	30.8	44.4	37
1.0×10^{-4}	80	25.5	43.3	50
	86	25.5	43.6	50
	91	25.5	43.9	51
	95	25.5	44.1	51
	100	25.5	44.3	50
5.0×10^{-5}	80	34.6	43.4	25
	84	34.6	43.6	25
	88	34.6	42.1	21
	91	34.6	42.2	21
	95	34.6	43.8	25
1.0×10^{-5}	80	28.9	43.0	40
	86	28.9	43.3	41
	91	28.9	43.5	40
	95	28.9	43.6	40
	98	28.9	43.8	40

Note: Spores were suspended in citrate buffer, pH 4 in each case.

D. STUDIES ON B. STEAROTHERMOPHILUS NCIB 10,003 (MUTANT) SPORES PREPARED FROM SULPHATE DEPLETED CULTURES (SO_4^-)

(a) The Effect Of Heating On The Dormancy Of Spores Prepared From Sulphate Depleted Cultures (SO_4^-)

Nutrient depletion studies of various spore kinds showed the extraordinary dormancy of SO_4^- spores (Figure 36). In this section of work, an attempt was made to activate the dormant spores for germination by heating.

Preliminary studies with the initial spore stock, stored at 4°C for 6 months, showed that dormancy was broken after prolonged storage. The percentage of dormant spores dropped from 87% to 10%. Consequently, a new batch of SO_4^- spores was prepared and the percentage of dormant spores redetermined. The new stock of SO_4^- spores contained 58.5% dormant spores as opposed to 87% obtained with previous batch of spores (see explanation below).

Sublethal heating of spore suspensions have been known to result in an increase in colony counts (Evans and Curran, 1943). In the present study, newly prepared SO_4^- spores were subjected to heating temperatures of 79.5 to 110°C for exactly 5 minutes and the colony count determined. The results are shown in Table 37. The unheated control suspension showed 4.188×10^7 colony counts/ml. and contained 58.5% dormant spores (measured w.r.t. total chamber count). Heating for 5 minutes at progressively lower temperatures than 97.3°C resulted in a progressive fall in the colony count from 11.7% (measured w.r.t. the colony count of the unheated control) at

TABLE 37

HEAT INDUCED CHANGES IN COLONY COUNT OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED IN SULPHATE DEPLETED CULTURES

TEMPERATURE USED (°C)	COLONY COUNT (x 10^7 counts/ml.)	* % CHANGE IN COLONY COUNT	** % HEAT ACTIVATION
79.5	2.000	-52.2	0
86.8	2.670	-36.2	33.5
91.3	3.160	-24.5	58.0
95.0	3.700	-11.7	85.0
97.3	4.998	+19.3	149.9
100.0	5.915	+41.2	195.8
102.0	6.506	+55.3	225.3
104.0	6.522	+55.7	226.1
106.0	5.912	+41.2	195.6
108.0	4.592	+ 9.6	129.6
110.0	5.370	+28.2	168.5

* Measured w.r.t. unheated control which gave 4.188 x 10^7 colony counts/ml.

** Colony count at 79.5°C arbitrary taken as control.

95°C to 52.2% at 79.5°C. On the other hand, when these spores were heated at temperatures of 97.3 to 104°C, there was a progressive increase in the colony count. The percentage of increase was 19.3% (again measured w.r.t. the colony count of unheated control) at 97.3°C to 55.7% at 104°C. When heated at temperatures above 104°C, the number fell progressively indicating that the effect of heat inactivation had override the effect of heat activation.

The data showed three phenomena summarised as follows:-

(1) Heat induced dormancy at heating temperatures of 95°C and below.

(2) Heat activation > heat inactivation (if any) at temperatures of 97.3 to 104°C.

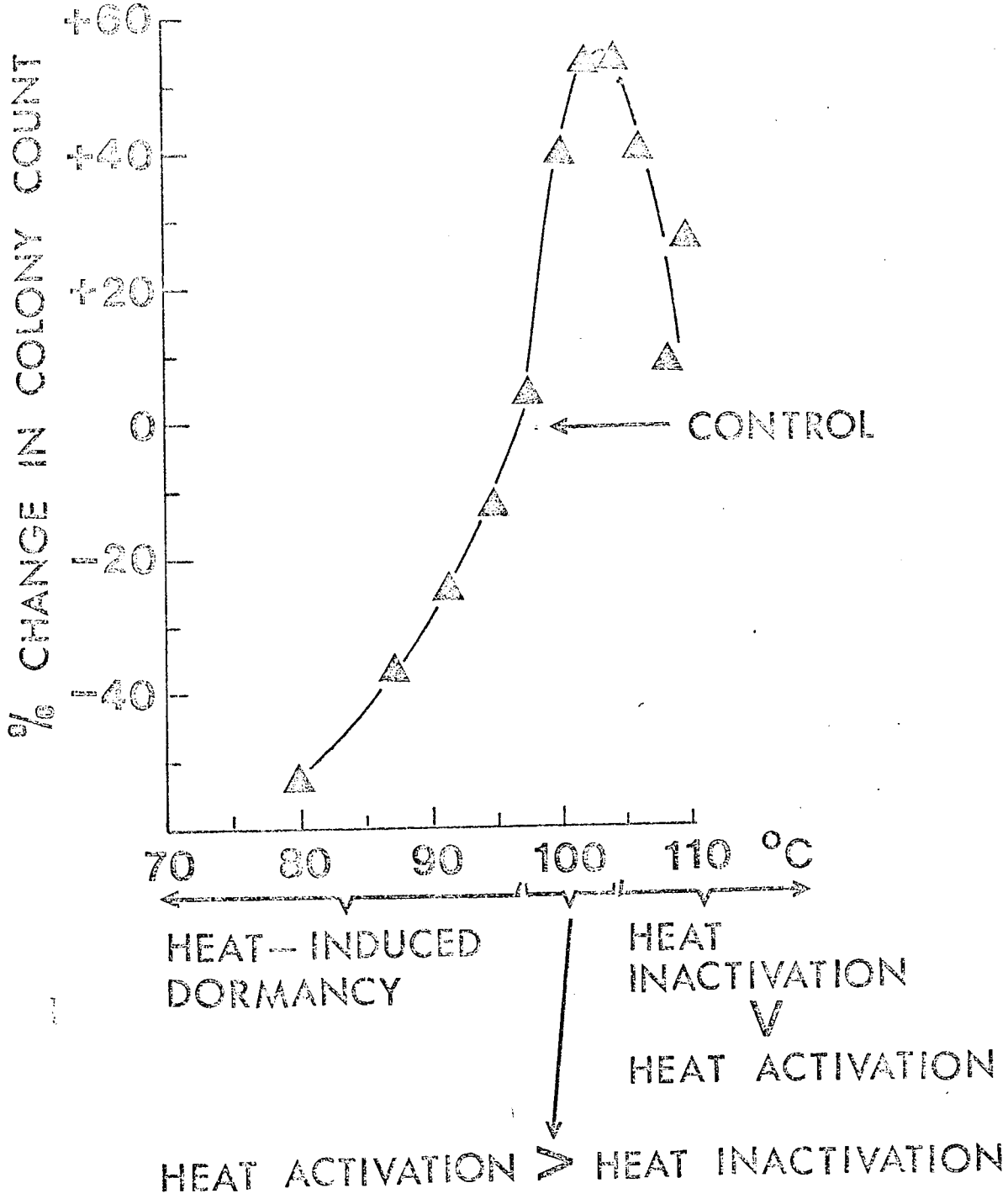
(3) Heat inactivation > heat activation at heating temperatures of 105°C and above.

The effects of heat induced change are best illustrated by Figure 67. The relationship between percentage changes in colony count and the temperature of heating was curvilinear rather than a straight line response. The data suggested that colony count changes would be minimal if the storage temperature was 96.5°C, this is the temperature when heat activation equals heat induced dormancy.

The spore stock used was obtained following batch cultivation at 60°C (see Section 2.F.). It is conceivable that some degree of dormancy was induced on the spores formed at this incubation temperature. The variation in the percentage of dormant spores

FIGURE 67

HEAT INDUCED CHANGES IN THE COLONY COUNTS OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED FROM SULPHATE DEPLETED CULTURES


% CHANGE IN COLONY COUNT
+60
+40
+20
0
-20
-40
CONTROL
70
80
90
100
110
°C
HEAT-- INDUCED DORMANCY
HEAT INACTIVATION
V
HEAT ACTIVATION
HEAT ACTIVATION > HEAT INACTIVATION

observed from batch to batch could well be the result of different degree of exposure to 60°C of spores formed after the completion of sporulation. Since 5 minutes heating at 79.5°C could induce a 52.2% drop in colony count, it is not unreasonable to assume that the batch to batch variations in the duration of exposure to 60°C of spores formed following sporulation is even greater than the experimental 5 minutes, giving rise to great variability of percentage dormant spores. This variation did not preclude the variation resulting from the method of harvesting and cleaning of spores discussed previously.

The fact that dormancy was broken on storage at 4°C for six months showed that dormancy was not maintained in SO_4- spores. It was lost on aging and this loss of dormancy through ageing operated by mechanisms unrelated to heat-induced dormancy.

Heat induced dormancy is different from thermal death because the dormant spores can be re-activated. Table 38 shows the results of keeping SO_4- spores in:-

(1) Dormancy inducing temperature (79°C) for 5 minutes.

(2) Dormancy inducing temperature for 5 minutes, cooled and followed by activating temperature (102°C) for another 5 minutes.

It can be seen that the colony counts were reduced following incubation at dormancy inducing temperature for 5 minutes. The colony counts were increased if these spores were returned to the activating temperature of 102°C for another 5 minutes.

An Arrhenius plot for the heat activation of SO_4- spores was

TABLE 38

HEAT INDUCED DORMANCY IN B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED FROM SULPHATE DEPLETED CULTURES AND REACTIVATION OF SPORES WITH HEAT INDUCED DORMANCY

TREATMENT	SAMPLE NUMBER	COLONY COUNT ($\times 10^7$ counts/ml.)	* % VIABILITY	% OF UNHEATED CONTROL	COMMENT
Unheated control	1	4.172	40.5		
	2	4.186	40.6		
Heated at 79°C for 5 mn.	1	3.254	31.6	77.9	Heat induced dormancy.
	2	3.008	29.2	72.0	
Heated at 79°C for 5 mn. Cooled. Reheated at 102°C for another 5 mn.	1	6.016	58.4	144.0	Reactivation of spores with heat induced dormancy.
	2	6.072	59.0	145.3	

* Measured w.r.t. total chamber count = 1.030×10^8 spores/ml.

performed by arbitrary taking the colony count at 79.5°C as a control count. The percentage increase in colony count with progressive higher heating temperature (heat activation) was calculated. The results are shown in Table 37 and plotted in Figure 68. It can be seen that linear relationship between log. k and $\frac{1}{T}$ was obeyed up to the heating temperature of 104°C, after which the effect of inactivation overrode the effect of heat activation resulting in a progressive fall in the readings of log. k with increase in heating temperatures. The activation energy and other thermodynamic data were determined as described before (Table 39). The thermodynamic properties of heat activation were: Ea = 35.4 k cal./mol.; ΔG = 39.2 to 39.5 k cal./mol.; ΔS = 11 cal./mol./deg. The activation energy was lower than the value of 46 k cal./mol. obtained with B. stearothermophilus NCIB 8919 spores (Brown and Melling, 1973) but higher than those required by the endospores of B. subtilis (Ea = 27.9 k cal./mol.; ΔG = 25.1 to 26.4 k cal./mol.; ΔS = 4.6 to 8.1 cal./mol./deg., Busta and Ordal, 1964). Levinson and Hyatt (1969) measured activation as the increase of germination rate over that of dormant spores. Spores of B. megaterium were activated throughout the range of test temperatures of 50 to 75°C and the activation energy was calculated to be 72.4 k cal./mol. Grecz and Olejniknow-Kurotza (1966) found the activation energy of Cl. botulinum spores to be 42 k cal./mol. by colony counts. The data of Keynan et al.(1964) for the activation of B. cereus T spores was calculated by Levinson and Hyatt (1969) to be 32 k cal./mol. There may be differences between spores of different species but there is the possibility that the difference is due to the different

FIGURE 68

ARRHENIUS PLOTS OF D.P.A., CALCIUM, MAGNESIUM AND MANGANESE RELEASE AND COLONY COUNT INCREASE OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED FROM SULPHATE DEPLETED CULTURES

		ACTIVATION ENERGY (k cal./mol.)
▽—▽	D.P.A. release	34.5
□—□	Calcium release	42.4
◇—◇	Magnesium release	11.1
△—△	Manganese release	19.2
○—○	Colony count increase	35.4

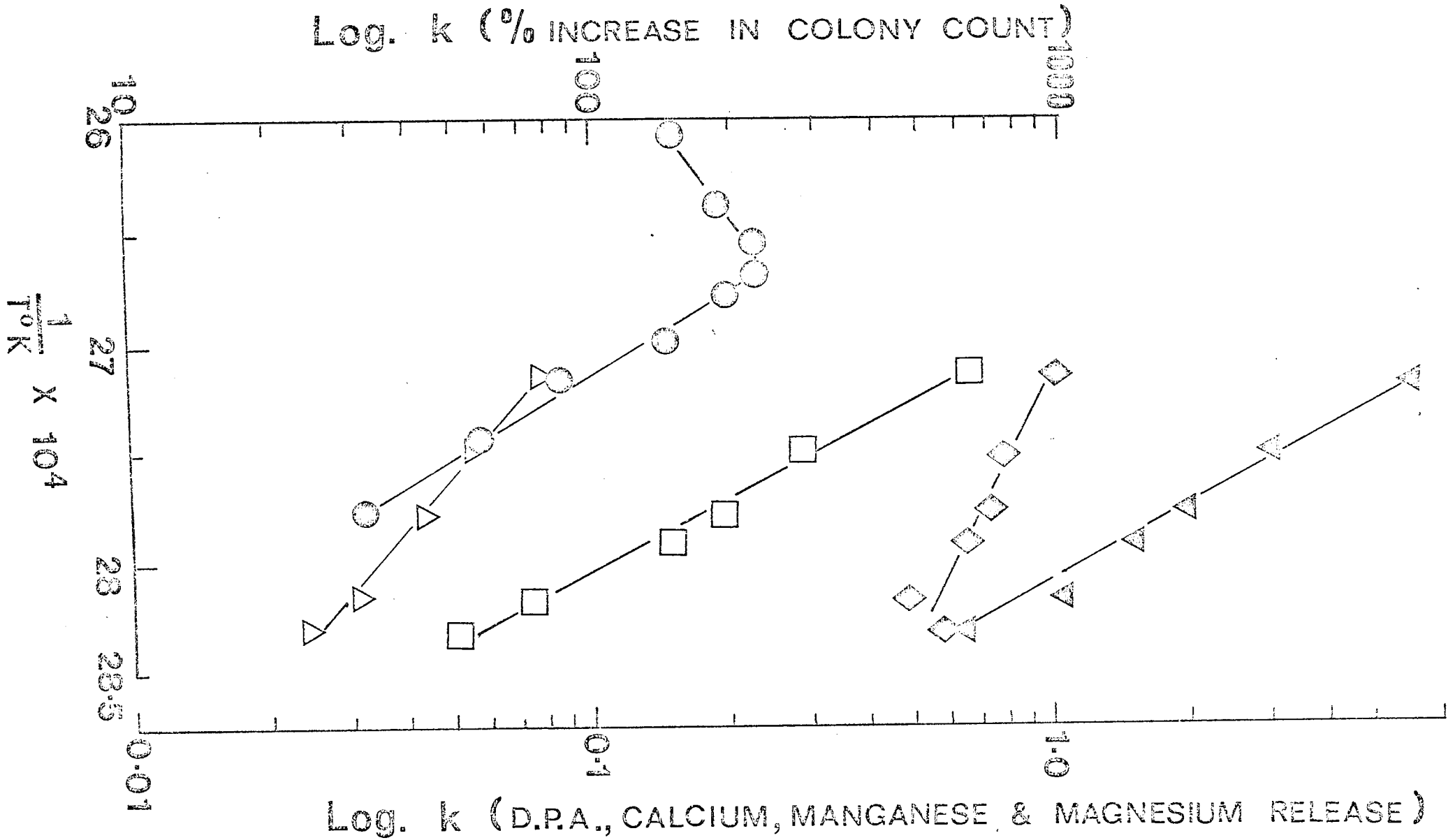

Log. k (% INCREASE IN COLONY COUNT)
1000
1000
2.9
2.8
2.7
2.6
2.5
$\frac{1}{T^{\circ}K} \times 10^4$
0.0
1.0
2.0
Log. k (D.P.A., CALCIUM, MANGANESE & MAGNESIUM RELEASE)

TABLE 39

THERMODYNAMIC FUNCTIONS FOR THE HEAT ACTIVATION OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED IN SULPHATE DEPLETED CULTURES

ACTIVATION TEMPERATURE (°C)	ACTIVATION ENERGY (k cal./mol.)	FREE ENERGY OF ACTIVATION (k cal./mol.)	ENTROPY OF ACTIVATION (cal./mol./deg.)
86.8	35.4	39.2	11
91.3	35.4	39.3	11
95.0	35.4	39.5	11
97.3	35.4	39.3	11
100.0	35.4	39.4	11
102.0	35.4	39.5	11

methods used (colony formation as opposed to increase germination rate).

(b) Kinetics Of Dipicolinic Acid, Calcium, Magnesium And Manganese Release From Spores Prepared From Sulphate Depleted Cultures (SO_4^-)

The method described in Section 6.C.(f) was used to follow the kinetics of release of D.P.A., calcium, magnesium and manganese from SO_4^- spores. The results are shown in Table 40. Arrhenius plots for D.P.A., calcium, magnesium and manganese release are shown in Figure 68. The slope of each line was determined by regression analysis and the thermodynamic data calculated (Table 41). The data suggested that the activation energy for the release of D.P.A. (34.5 k cal./mol.) was similar to that for the heat activation of these spores (35.4 k cal./mol.) (see above). However, activation energies for the release of calcium, magnesium or manganese were unrelated to the heat activation of these spores.

The results confirmed the observation of Brown and Melling (1973) that the apparent activation energies for the release of D.P.A. and colony count increase (heat activation) were similar. The activation energy for the release of D.P.A. from SO_4^- spores was similar to those obtained with N- spores, which showed 95% germination without prior heat activation. This low percentage of dormant spores (5%) in N- spores prevented the study of dormancy breaking using the colony count method described above. Attempts to study dormancy breaking by measuring the increase in germination rate (Levinson and Hyatt, 1969) were unsucessful because of the lack

TABLE 40

THE RELEASE OF DIPICOLINIC ACID, CALCIUM, MAGNESIUM AND MANGANESE FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED IN SULPHATE DEPLETED CULTURES

RELEASE TEMP. (°C)	RELEASE OF: D.P.A. (µg./*ml./mn.)	CALCIUM ($\times 10^{-1}$ µg./*ml./mn.)	MAGNESIUM ($\times 10^{-1}$ µg./*ml./mn.)	MANGANESE ($\times 10^{-3}$ µg./*ml./mn.)
80	0.64	0.52	0.58	2.40
82	1.07	0.75	0.48	3.12
85	1.53	1.50	0.65	--
88	1.93	1.92	0.72	4.40
91	3.00	2.88	0.77	5.50
95	5.60	6.50	1.03	7.92

Note: Spores were suspended in citrate buffer, pH 4 in each case.

* Spore concentration normalized to a value of 2.82×10^{8} spores/ml. in each case.

of precision in the method used. Whether or not sublethal heating induced dormancy in N- spores was not studied, the effect was not realised at the time N- spores were studied.

The activation energies for the release of magnesium and manganese from SO_4- spores were lower than those obtained with N- spores (Tables 35 and 36), showing that the method of spore cultivation affected the result of the Arrhenius plot. The activation energy, entropy of activation and free energy of activation values were all in the range expected of enzymatic reactions.

TABLE 41

THERMODYNAMIC FUNCTIONS FOR THE RELEASE OF DIPICOLINIC ACID, CALCIUM, MAGNESIUM AND MANGANESE FROM B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES OBTAINED IN SULPHATE DEPLETED CULTURES

RELEASE OF:	RELEASE TEMP (°C)	ACTIVATION ENERGY (k cal./mol.)	FREE ENERGY OF ACTIVATION (k cal./mol.)	ENTROPY OF ACTIVATION (cal./mol./deg.)
D.P.A.	80	34.5	40.1	16
	82	34.5	40.0	15
	85	34.5	40.1	16
	88	34.5	40.3	16
	91	34.5	40.3	16
	95	34.5	40.3	16
Calcium	80	42.3	41.9	1
	82	42.3	41.9	1
	85	42.3	41.7	1
	88	42.3	41.9	1
	91	42.3	42.0	1
	95	42.3	41.8	1
Magnesium	80	11.1	41.8	87
	82	11.1	42.2	88
	85	11.1	42.2	87
	88	11.1	42.4	87
	91	11.1	43.0	88
	95	11.1	43.0	87
Manganese	80	19.2	44.0	70
	82	19.2	44.1	70
	88	19.2	44.6	70
	91	19.2	48.2	80
	95	19.2	48.4	79

Note: Spores were suspended in citrate buffer, pH 4 in each case.

7. THE RELATIONSHIP BETWEEN CHEMICAL COMPOSITION AND HEAT RESISTANCE

Murrell and Warth (1965) analyzed the calcium, magnesium, dipicolinic acid, diaminopimelic acid and hexosamine contents of 20 spore preparations of Bacillus species, with a 700-fold range in heat resistance and reported that calcium showed a small but significant ($P < 0.01$) increase with heat resistance. Dipicolinic acid did not increase significantly with heat resistance. Diaminopimelic acid increased, while magnesium and Mg/Ca molar ratio each decreased significantly ($P < 0.001$) with heat resistance.

In the present study, the content of dipicolinic acid, calcium, magnesium, manganese and Mg/Ca molar ratio of B. stearothermophilus NCTC 10,003 (mutant) spores were determined with preparations obtained under different cultivation conditions (data pooled from the results obtained in Section 6.A. to 6.D.) These spores showed varying degrees of heat resistance, measured as log. D_3 at 110°C (the time taken to destroy 90% of the initial spore population i.e. from time zero. See Section 6.A.). Attempts were made to determine the correlation between the content of these constituents and spore heat resistance.

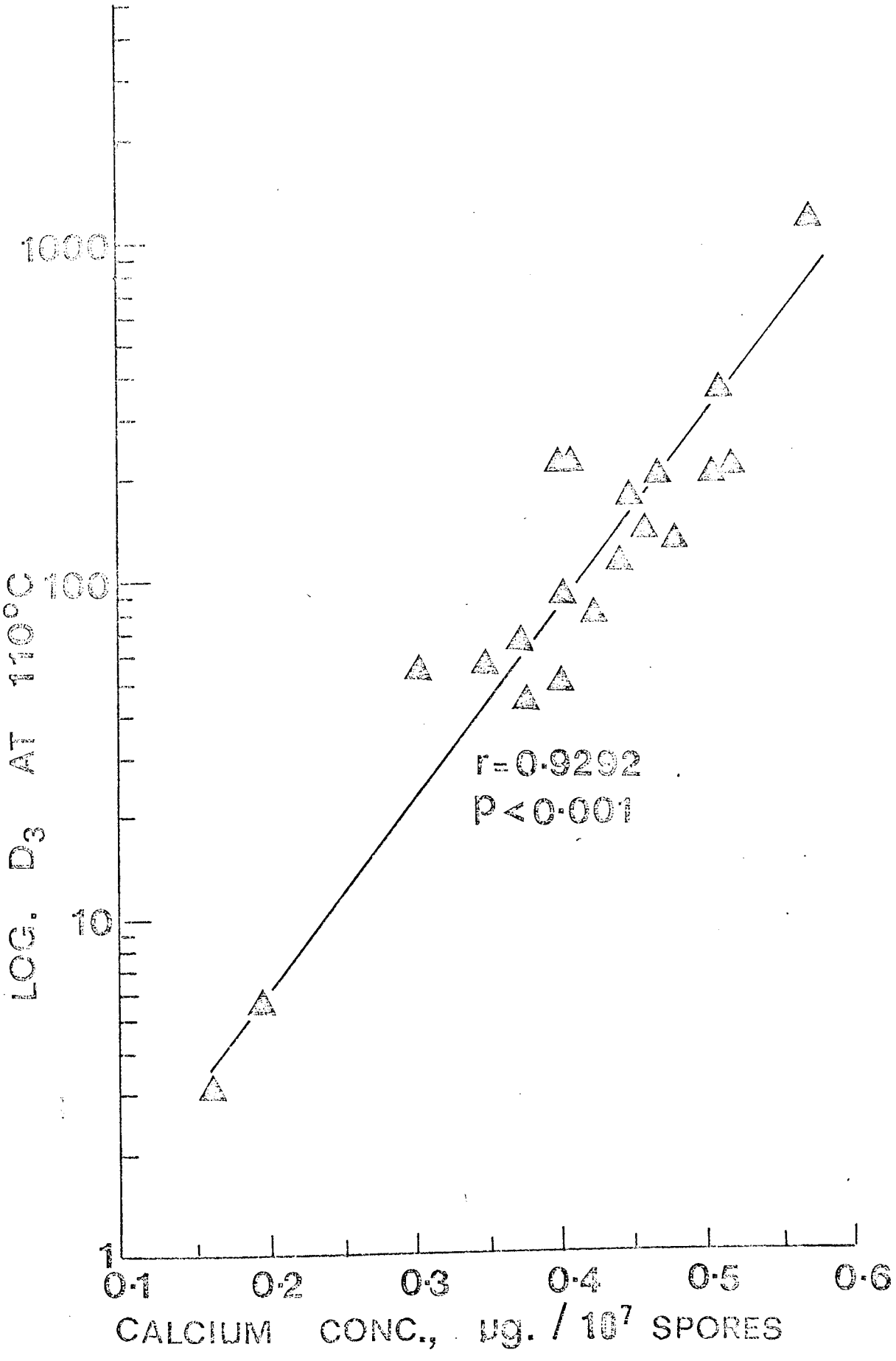
A. THE RELATIONSHIP BETWEEN THE CONTENT OF CALCIUM, DIPICOLINIC ACID OR MAGNESIUM AND HEAT RESISTANCE OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES PREPARED IN DIFFERENT SPORULATION MEDIA

Figure 69 shows the relationship between log. D_3 and calcium

FIGURE 69

THE RELATIONSHIP BETWEEN HEAT RESISTANCE AND CALCIUM CONTENT OF B. STEAROTHERMOPHILUS NCTC 10,003 (NUTANT) SPORES

Note: Data pooled from the results obtained with the various variety of spores studied (Section 6).


1000
100
10
1
LOG. D3 AT 110°C
r=0·9292
p<0·001
0·1
0·2
0·3
0·4
0·5
0·6
CALCIUM CONC., µg. / 10⁷ SPORES

content of spores. A significant correlation ($P < 0.001$) was obtained.

Heat resistance was also significantly correlated to dipicolinic acid content ($P < 0.001$) (Figure 70). This finding agreed with the report of Church and Halvorson (1959), they showed the heat resistance of B. cereus T spores varied directly with dipicolinic acid content. However, Murrell and Warth (1965) could not find any quantitative relationship between heat resistance and dipicolinic acid content. This discrepancy may be due to the fact that these workers used several species of bacteria in their studies. It is probable that the dipicolinic acid content between species does not vary to the same extent as the dipicolinic acid content of a single specie grown under different cultivation conditions.

Magnesium content showed a significant inverse relationship with spore heat resistance ($P < 0.001$) (Figure 71). It should be pointed out that only those readings that showed a magnesium concentration of 0.005 to 0.08 $\mu g./10^7$ spores were shown. There were several readings in the data that lay outside this range, with spores from medium high in magnesium (5×10^{-3} M.) showing a concentration of 7.7195 $\mu g./10^7$ spores (Table 26). PO_4- spores prepared from medium buffered with HEPES were also characteristically high in magnesium (Tables 22, 27). This high level of magnesium probably represent contamination of superficially bound counter-cations described by Murrell and Warth (1965). The contamination arose because of the exceptional high level of magnesium present in the sporulation media of these spores. Murrell and Warth (1965) found that such

FIGURE 70

THE RELATIONSHIP BETWEEN HEAT RESISTANCE AND D.P.A. CONTENT OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES

Note: Data pooled from the results obtained with the various variety of spores studied (Section 6).

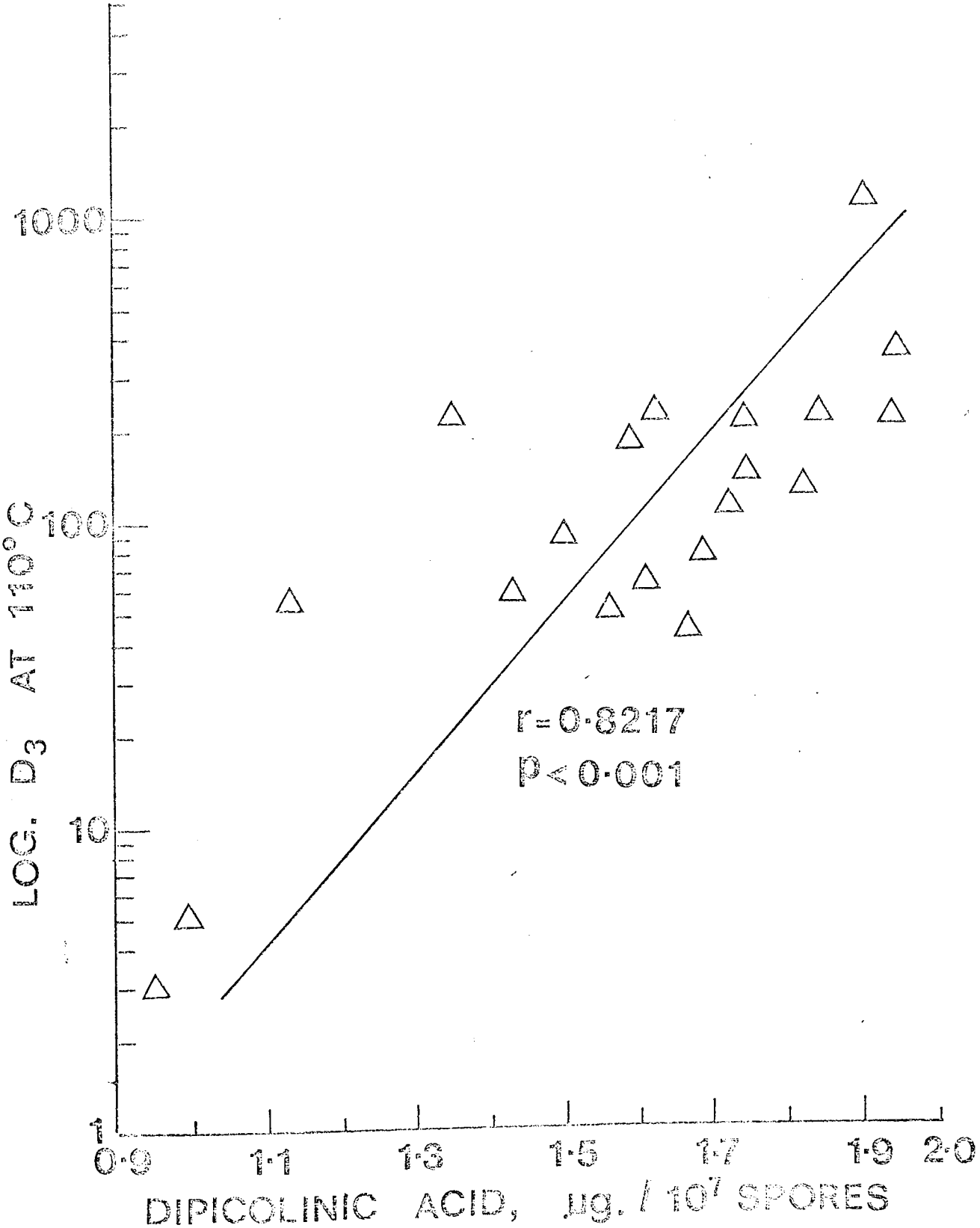
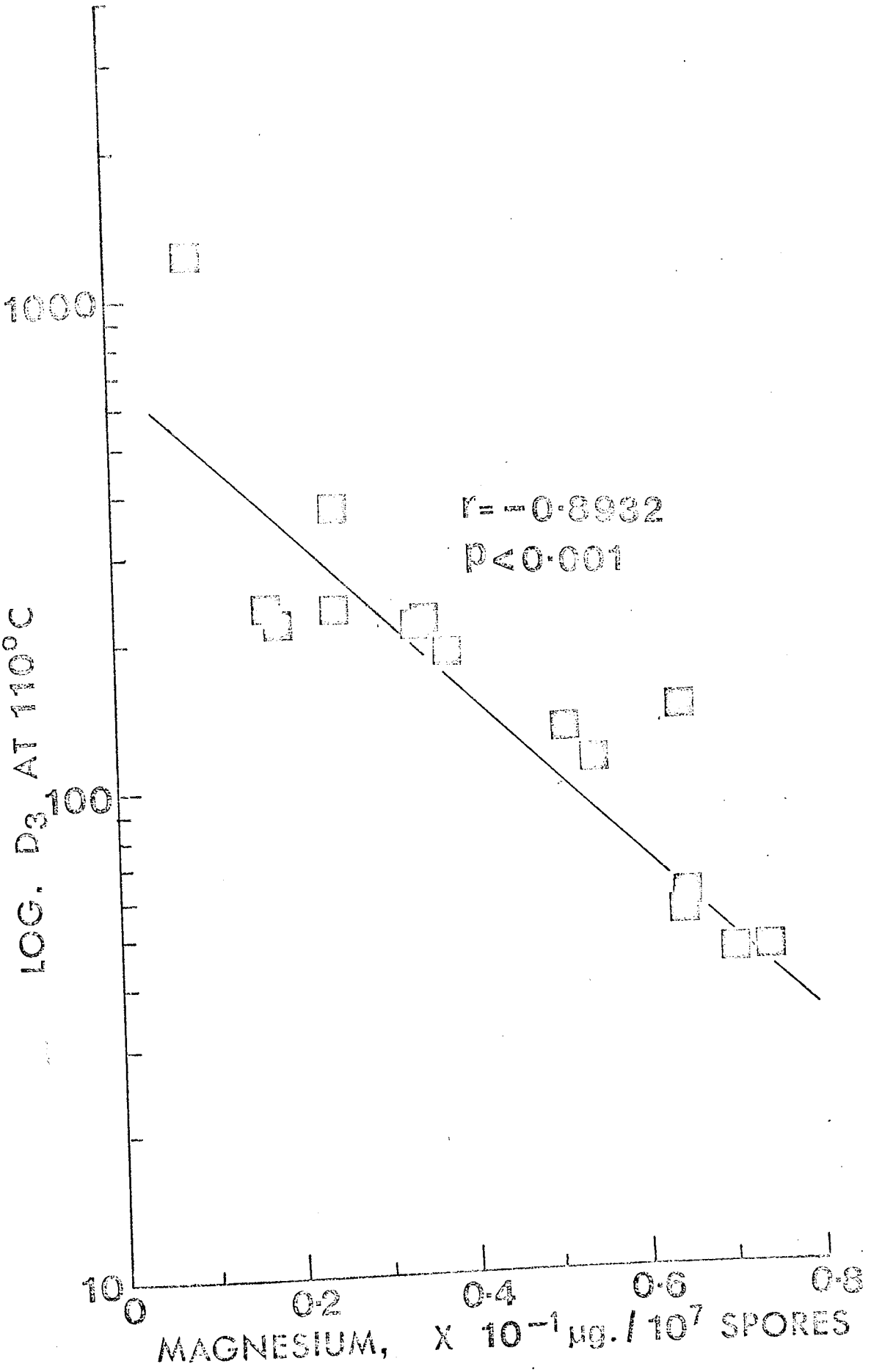

1000
100
10
1
LOG. D_3 AT 110° C
r = 0·8217
P < 0·001
0·9
1·1
1·3
1·5
1·7
1·9
2·0
DIPICOLINIC ACID, µg. / 10^7 SPORES

FIGURE 71

THE RELATIONSHIP BETWEEN HEAT RESISTANCE AND MAGNESIUM CONTENT OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES

Note: Data pooled from the results obtained with the various variety of spores studied (Section 6).


1000
100
10
LOG. D_3 AT 110°C
r = -0·8932
P<0·001
0
0·2
0·4
0·6
0·8
MAGNESIUM, X 10^{-1} µg. / 10^7 SPORES

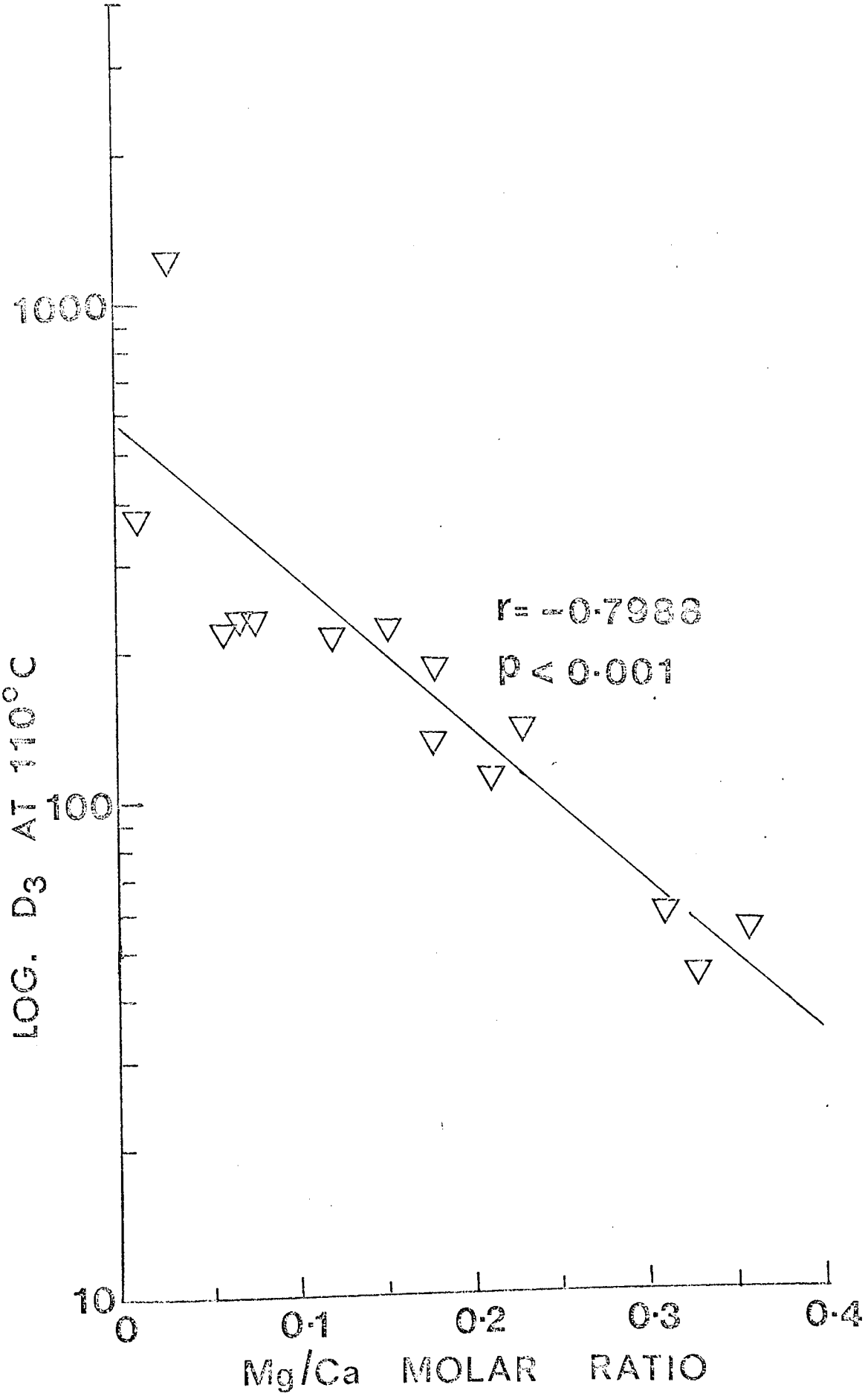
contamination was rapidly removed during acid washing at 5°C and the removal of contaminating cations did not affect the heat resistance of the resulting spores. With the exception of reading from spores with 7.7195 µg. $Mg^{2+}/10^7$ spores and those from HEPES buffered media, the inclusion of other readings higher than 0.08 µg./10^7 spores did not invalidate the relationship found. Conceivably, if spores with surface bound contaminating Mg^{2+} had been acid-washed in a manner suggested by Murrell and Warth (1965). The magnesium content of the resulting acid-cleaned spores might fall within the range expected of significant correlation between magnesium content and spore heat resistance.

The Mg/Ca molar ratio also show significant inverse relationship with heat resistance ($P<0.001$)(Figure 72). The molar ratio values generally vary from 0.025 to 0.4. Readings greater than 0.4 were not shown in Figure 72. These readings were obtained from those spores high in magnesium content (surface contamination) described above.

FIGURE 72

THE RELATIONSHIP BETWEEN HEAT RESISTANCE AND MAGNESIUM/CALCIUM MOLAR RATIO OF B. STEAROTHERMOPHILUS NCTC 10,003 (MUTANT) SPORES

Note: Data pooled from the results obtained with the various variety of spores studied (Section 6).


1000
100
10
LOG. D_3 AT 110°C
r = −0·7986
p < 0·001
0
0·1
0·2
0·3
0·4
Mg/Ca MOLAR RATIO

DISCUSSION

1. NUTRITIONAL REQUIREMENTS OF B. STEAROTHERMOPHILUS

With the exception of B. stearothermophilus NCTC 10,003 (mutant), all other strains studied showed an absolute methionine requirement (Table 12). Such results are in agreement with those previously reported by Campbell and Williams (1953) and Bhat and Bilimoria (1955). O'Brien and Campbell (1957) later showed that minimal requirements for fermentation, outgrowth of spores and vegetative cell growth of B. stearothermophilus and B. coagulans included methionine.

Campbell and Sniff (1959) showed that folic acid is required by B. coagulans grown at 45°C. At 55°C, the influence of folic acid in methylation seems to be lost as shown by its absolute requirement for methionine. The effect of temperature on the nutritional requirements of thermophilic species was later confirmed by Campbell and Pace (1968). Consequently, the absolute requirements listed in the present study may not be valid at temperatures other than 55°C.

The interchangeability between methionine and folic acid mentioned was also shown by the methionine-requiring mutant of E. coli 113-3, which is widely used as an assay organism for cobalamin, the vitamin required in the biosynthesis of methionine. Thermophiles showing an absolute methionine requirement would also be useful for cobalamin assays. Growth would be accelerated at 55°C compared with 37°C. A growth depletion study with methionine showed that under proper control experimental conditions, a

sensitivity of 10^{-5} M. can be readily achieved (Figure 18).

The absolute requirement for biotin, nicotinic acid and thiamin shown by B. stearothermophilus NCIB 8157, 8919, 8920 and NCTC 10,003 (wild) showed that B. stearothermophilus are nutritionally far more homogeneous than mesophiles (Knight and Proom, 1950; O'Brien and Campbell, 1957). The results obtained in the present study were strikingly uniform. This is in contrast to those reported by Campbell and Williams (1953). These workers found some variability in the nutritional requirements of eight strains of Bacillus stearothermophilus studied, even when the incubation temperature was uniformly kept at 55°C. It should be pointed out that these workers made their studies with stationary tube cultures as opposed to shaking cultures used here. Whether or not the degree of culture aeration determines the nutritional requirements is not known. Oxygen is known to be one-half as soluble at 55°C as at 30°C (Allen, 1950; 1953).

The absolute requirement for biotin shown here has previously been demonstrated by Campbell and Williams (1953) in B. coagulans and B. stearothermophilus. These workers also found that all biotin requiring strains could also utilize oxybiotin to satisfy this requirement. With the exception of one strain of B. coagulans which utilizes desthiobiotin only at 36°C but not at 45°C or 55°C, all other strains could also utilize desthiobiotin. Some strains could satisfy their biotin requirement with aspartic acid, some with oleic acid and some with pimelic acid. All these compounds represent normal intermediates in the chain of synthetic reactions

by which microorganisms make biotin:

x ⟶ pimelic acid ⟶ desthiobiotin ⟶ biotin

Each of these steps represents an unknown number of enzymatic reactions taking place in those microorganisms which can synthesize biotin, such as most of the mesophiles (White, 1972). The results suggest the heat lability of part of the biotin synthetic reactions.

Nothing is known of the biochemical and genetic means by which B. stearothermophilus NCTC 10,003 (mutant) developed the ability to grow without added amino acids or vitamins. A similar prototrophic strains of B. stearothermophilus was isolated by Epstein and Grossowicz (1969). The prototrophic nature of B. stearothermophilus NCTC 10,003 (mutant) did not change over the entire range of temperatures of 45 - 65°C, suggesting that under the conditions tested, all its biosynthetic activities are thermostable. Another, less likely possibility is that alternative biochemical pathways operate at the different incubation temperatures employed.

The metabolic interaction of L-valine and L-leucine obtained in the present study (Figure 15) had been reported by several workers. Tatum (1964) found L-valine inhibited the growth of E. coli K12, this inhibition was overcome by L-isoleucine and its 6-carbon precursors (Umbarger and Brown, 1955). Leahy (1968) showed that the diauxic lag between growth on glucose and lactose in E. coli K12 was extended by L-valine and that this extended lag was shortened by L-isoleucine. This variation in the length of diauxic lag was used as an assay procedure for L-valine or L-isoleucine with sensitivities of about 0.17 mμ mole/ml. or 0.04 mμ mole/ml.

respectively. Piperno and Oxender (1968) showed that one transport system exists in E. coli K12 for isoleucine, leucine and valine. In the case of B. stearothermophilus NCIB 8919, it seems that L-leucine alone is inhibitory to growth whereas L-valine is not (Figure 15). When both compounds were present in the growth medium, there may be competition for entry into the cells due to a common transport system (as suggested by Piperno and Oxender, 1968) giving rise to low concentration of L-leucine in the cells so that growth took place normally. This explains the occurrence of growth in the presence of both compounds. Presumably, when both compounds were absent from the growth medium, growth occurred because the inhibitory effect of L-leucine was removed.

2. THE USE OF CHEMICALLY DEFINED MEDIUM

Although several defined and minimal liquid media for the growth of B. stearothermophilus have been reported (Campbell and Williams, 1953; Welker and Campbell, 1963; Anderson and Friesen, 1972; Rowe et al., 1975). All the media described (except Anderson and Friesen, 1972) used glucose-mineral salts supplemented with a long list of vitamins and amino acids which were found to be partly unnecessary. The medium formulated in Table 13 is able to support good growth of B. stearothermophilus strains NCIB 8920, 8919 and NCTC 10,003 (wild) despite its simplicity.

The medium described by Anderson and Friesen (1972) was adequate for the growth of B. stearothermophilus NCTC 10,003 (mutant) but did not support the growth of other strains studied. This medium employed ammonium phosphate buffer in high concentration, giving rise to a high background contamination of magnesium and sulphate. The medium was subsequently modified by replacing ammonium phosphate buffer with lower concentration of sodium potassium phosphate buffer (Table 14).

Although manganese, iron and calcium are probably necessary for growth of all bacteria, they are required in such trace amounts that they need not be added to the defined medium.

The lack of a simple defined medium has greatly restricted genetic and physiological studies of B. stearothermophilus. The media reported here are minimally defined in a quantitative sense. These media are suitable for mass cultivation using a chemostat in

which one or more components are present in sufficiently low concentration to limit the size of cell population.

3. GROWTH DEPLETION STUDIES

The shape of the growth curve is in several cases characteristic of the nature of the nutrient depletion. Growth depletion by carbon results in a rapid cessation of growth, followed by some degree of cell lysis (Figures 17, 27 and 30). Energy is generated during growth and is necessary to maintain the selective permeability of the cytoplasmic membrane. Rapid depletion of the energy source due to glucose (carbon) depletion may result in lysis of the sensitive fraction of the population due to an inability to maintain the required internal ionic environment. The susceptibility to lysis is undoubtedly determined by the levels of endogenous energy reserves.

Nitrogen depleted growth, on the other hand, is characterised by the immediate cessation of growth and the maintenance of maximum optical density readings, indicating that the population size was unchanged under this condition (Figures 24, 29 and 32). Macrae and Wilkinson (1958) found nitrogen deficient B. megaterium accumulated a high content of poly-β-hydroxybutyric acid (P.H.B.) and autolysis was delayed. B. stearothermophilus studied here did not appear to accumulate P.H.B. following nitrogen depletion. The accumulation of P.H.B. in cells can be detected microscopically using phase contrast microscope; the P.H.B. inclusion showed up as refractile bodies. The possibility that other compounds may be accumulated under nitrogen depleted condition cannot, however, be excluded. Of particular interest is the report by Slock and Stahly (1974), who observed that an intracellular glucose

containing polysaccharide accumulates in B. cereus when the accumulation of P.H.B. was prevented. The polysaccharide was implicated to serve both as a carbon and an energy storage compound during sporulation. The compound accumulated in considerably greater quantities in cells grown in media in which growth was depleted by a nitrogenous compound (N-medium) than in media in which growth was depleted by glucose (G-medium).

In the case of vitamin, methionine or ion depleted growth, the optical density continued to increase slowly at the onset of depletion (Figures 19, 20, 21, 22, 26, 33, 34 and 36). This may be in part due to changes in cell morphology and sporulation. Morphological and chemical transformation following nutrient depletion have been reported with B. licheniformis (Forsberg et al., 1973) and A. globiformis (Chan et al., 1973). The slow increase in optical density may also represents the adaptation of the cells to the nutrient depleting condition. Adaptation by B. megaterium following magnesium depleted growth has been previously reported (Brown and Hodges, 1974). This took the form of biphasic growth.

Another salient feature found in several depletion experiments was the variation in growth rate of cultures containing different concentrations of depleting nutrient (for example, Figures 17, 19, 20, 22, etc.). The culture containing the lowest concentration of depleting nutrient often exhibited the lowest growth rate. The growth rate progressively increased with progressive increase in the concentration of depleting nutrient until the maximum growth rate was achieved. Stanier et al. (1971) reported

relationship between glucose concentration and growth rate of E. coli at low glucose concentration. It was reported that a concentration of 0.22×10^{-4} M. glucose was sufficient to support growth of E. coli at half the maximal rate and the concentration range over which changes in growth rate may be demonstrated was of the order of 10^{-5} M. for carbon and energy sources, and even lower for minerals and growth factors.

Monod (1942) suggested that the relationship between substrate concentration and growth rate is governed by a Michaelis-Menten type equation used in enzymology:

$$U = U_{max.} \frac{S}{K_s + S}$$

where U is the growth rate of the culture;

$U_{max.}$ is the maximum value of U;

S is the growth limiting substrate concentration;

K_s is a constant which is numerically equal to the value of S when $U = \frac{1}{2} U_{max.}$

This equation is based on assumption that a reaction will take place when two reacting substances (substrate and enzyme) collide in solution and are held close together at appropriate orientation and juxtaposition with respect to each other. Consequently, U value (growth rate or enzymic reaction rate) will be low at very low S value (substrate concentration). The value of U increases proportionally as the value of S increases until a maximum value of U equal to $U_{max.}$ is reached. The decrease of growth rate at low concentration of depleting nutrient may be attributed to the sub-saturation of the

transport system (enzyme in the case of enzymology) which enabled passage of the substrate into the cell.

The concentration of depleted nutrient required to support growth to an identical optical density was higher with Bacillus stearothermophilus than was with B. megaterium (Brown and Hodges, 1974). For example, to support growth to E_{420} of 0.4, a glucose concentration of 1.0×10^{-3} M., 1.7×10^{-3} M., 1.3×10^{-3} M. was required for B. megaterium, B. stearothermophilus NCIB 8919 and B. stearothermophilus NCTC 10,003 (mutant) respectively. Similarly, to support growth to E_{420} of 1.0, magnesium concentration of 9.0×10^{-6} M. (first phase growth) and 3.0×10^{-6} M. (second phase growth) were required for B. megaterium whereas 1.9×10^{-5} M., 1.6×10^{-5} M. was required for B. stearothermophilus NCIB 8919 and B. stearothermophilus NCTC 10,003 (mutant) respectively. There may be some differences in the optical property of the different bacteria, but the difference in concentration required was too large to be accounted by the difference in the optical property alone.

Coultate and Sundaram (1975) studied the energetics and molar growth yields of a prototrophic strain of B. stearothermophilus at different incubation temperatures. They found the molar growth yield values of the thermophile, at lower growth temperatures were similar to those reported for aerobically grown mesophilic bacteria, both on glucose and on succinate. At progressively higher growth temperatures, the molar growth yields were progressively reduced. Under this condition, a lower proportion of glucose carbon was incorporated into the cells and a corresponding greater proportion

was left incompletely utilized in the medium, mostly as acetate. This suggests a greater inefficiency in the co-ordination of the non-oxidation and oxidation phases of glucose metabolism at higher incubation temperatures. Also, at high incubation temperature, there was uncoupling of energy production from respiration. Conceivably, similar wastage of glucose occurred in the present study. This may explain the higher concentration of glucose required by B. stearothermophilus when compared with B. megaterium. However, the higher demand for minerals of the thermophiles still remain unexplained. It seems likely that thermophiles may be structually far more mineral rich than the mesophiles.

The biphasic growth curve obtained in the presence of two carbon or nitrogen sources (Figure 30 and Figure 32), has also been previously reported by Monod (1942). Growth in this manner is termed diauxic. According to Paigen and Williams (1970), diauxic growth occurs whenever two essential conditions are met. First, adaptation to the less preferred or secondary carbon source is completely prevented in the presence of the preferred compound; and second the process of adaptation to the second substrate occurs under non-gratuitous conditions. Combinations of substrate which do not meet these requirements are used either simultaneously or sequentially with no intervening lag. B. stearothermophilus NCTC 10,003 (mutant) used glucose readily as a primary substrate. The utilisation of glutamic acid probably required the syntheses of some inducible enzymes during the period of lag.

The growth depletion data of thiamin, nicotinic acid and biotin

obtained with B. stearothermophilus NCIB 8919 suggest the feasibility of using the method for microbiological assay of these vitamins. The advantages of turbidimetric microbiological assay have been pointed out by Kavanagh (1960). The method is applicable to about as wide a range of growth factors as the plate method. Moreover, the answer obtained by a turbidimetric method is not affected by diffusion of the active substance as often encountered in plate method.

One of the perennial problems with microbiological assay is the preservation of the culture. Ideally, the organism should be from which the same population can be obtained easily and repeated over a long period. The sensitivities of the cells should follow the normal distribution curve. In the present study, the bacteria were kept as spore stock. It is generally accepted that the spores are far more genetically stable than the vegetative cells. Friesen and Anderson (1974) reported no change in spore heat resistance of B. stearothermophilus even after two years storage. Also, the fact that the assay could be done at 55°C made it more attractive, growth would be relatively rapid. The sensitivity of the assay would be in the range of 10^{-10} M. for biotin; 10^{-7} M. for nicotinic acid and 10^{-8} M. for thiamin.

4. FACTORS AFFECTING THE DEGREE OF SPORULATION

A. THE EFFECT OF CULTURE AERATION

The requirement of adequate aeration of cultures for optimum sporulation shown by B. stearothermophilus has been previously reported by Knaysi (1945) and Tinelli (1955). Oxygen is required for the oxidation of the accumulated organic acids by the T.C.A. cycle (Knaysi, 1945). Tinelli (1955) observed lysis and lack of spore formation with insufficient aeration of B. megaterium. With better aeration an increase in oxygen consumption occurred just before the appearance of spores. Later, the oxygen consumption declined.

Oxygen is known to be one-half as soluble at 55°C as at 30°C (Allen, 1950; 1953). In the present study, cultures were incubated at 60°C, the requirement for oxygen would be expected to be more critical at this high temperature.

B. THE EFFECT OF SPECIFIC NUTRIENT DEPLETION

The hypothesis that sporulation in bacilli is a response of cells to the depletion of nutrient necessary for growth was first proposed by Buchner(1890) and has since appeared many times in the literature (Knaysi, 1948; Grelet, 1957; Holmes and Levinson, 1967). Grelet (1957) showed that with B. megaterium strain MLA growing in synthetic medium, sporulation occurred when growth was depleted by glucose, nitrate, sulphate, iron or zinc, but did not occur when the depleting nutrient was potassium, magnesium or manganese. The results obtained with B. stearothermophilus NCTC 10,003 (mutant) support the findings

of Grelet (1957). Sporulation occurred at various degrees during carbon, nitrogen, sulphate or phosphate depletion of growth.

The high percentage sporulation occurred in sulphate depleted culture is surprising. Ordal (1957) found that when sulphur-containing amino acids (methionine and cysteine) were left out of the completed medium used to grow B. coagulans, sporulation was markedly reduced. When such medium was fortified with inorganic SO_4^{2-}, optimal sporulation was again achieved. Sulphate ion was also reported to be indispensible for the microcycle sporulation of B. megaterium, which also required the presence of phosphate ions (Holmes and Levinson, 1967). It was reported that sulphate was necessary for the rapid oxidation of glucose and the rapid utilization of the acids produced. In sulphate-deficient glucose-ammonia medium, the pH of the culture fell and rose slowly, but visible forespores were not observed. Whether or not the failure of sporulation was due to pH changes was not investigated. In the present study, the medium for sporulation was buffered with phosphate buffer or HEPES buffer and pH was maintained in the range of pH 7.0 to 7.2.

The observation that sporulation was inhibited upon magnesium depletion confirmed the previous report of Brown and Hodges (1974). The requirement for the presence of an excess concentration of magnesium for bacterial sporulation is not known. Magnesium has been reported to be required in the early stationary phase cells of B. megaterium during the process of commitment to sporulation (Greene and Slepecky, 1972). Magnesium is known to stabilize the

bacterial cytoplasmic membrane, either intact on free protoplasts or isolated as membrane fragments (Weibull, 1956). It is a required cofactor for many enzyme activities and is a key structural component of ribosomes. The role of magnesium in enzymic activities is replaceable by other metals, particularly by manganese (Dixon and Webb, 1964). However, its role in ribosomes is not replaceable by other metals. In as far as the protein metabolism of the sporulating cells is concerned, sporulation is known to be degradative as well as synthetic. About 18% of the total cellular protein were degraded during the sporulation of B. subtilis and extensive synthesis of new protein took place (Spudich and Kornberg, 1968). Kennell and Kotoulas (1967) found magnesium starved cells of A. aerogenes synthesized soluble proteins at 45% of the instantaneous rate per ml. of the cells growing exponentially at the start of the starvation. It seems at first glance, that the failure of magnesium depleted cultures to sporulate is due to the inability of the sporulating cells to synthesize adequate amounts of spore protein. This explanation, however, could not satisfactory explain the sporulation of cultures simultaneously depleted of magnesium and carbon source. A plausible explanation should perhaps, take into consideration the energy levels of the sporulating cells.

The reason for the difference in the extent of sporulation of cultures depleted in various ways remain speculative. Hanson and Mackechnie (1969) found that during nitrogen, adenosine or phosphate limited growth of B. subtilis and B. licheniformis, the syntheses of citrate synthase and aconitase were derepressed. On the other hand, sulphate and tryptophan limitation of growth, restrict anabolism but

allow the continued synthesis of the metabolite necessary for repression of the synthesis of the two enzymes. Moreover, it has been previously reported that there is an obligate requirement for the presence and activity of the enzymes of the Krebs tricarboxylic acid cycle in order for sporulation to occur (Hanson et al., 1963; Szulmajster and and Hanson, 1965). If this is true, then perhaps the observed difference in the extent of sporulation may be explained by the effects of depletion of specific nutrients on the syntheses of some crucial enzymes responsible for sporulation. Alternatively, specific nutrient depletion may affect the syntheses of some effectors of catabolite repression of sporulation.

C. THE EFFECT OF ADDED GLUTAMIC ACID

The apparent relationship between sporulation and glutamic acid concentration shown by B. stearothermophilus NCTC 10,003 (mutant) raised many interesting problems relative to sporulation control. Schaeffer et al. (1965) have reported on catabolic repression of bacterial sporulation and they stressed the importance of an intracellular level of a nitrogen-containing catabolite in repressing spore genes during growth of B. subtilis. Szulmajster and Hanson (1965) showed that the simultaneous presence of glucose and glutamic acid caused repression of aconitase synthesis in B. subtilis which in turn led to the inhibition of spore formation. Bacillus stearothermophilus NCTC 10,003 (mutant) studied here required a level of 2.4×10^{-3} M. glutamic acid for optimal sporulation (Table 21). Sporulation also occurred in the presence of excess carbon (glucose plus glutamic acid) and nitrogen source (NH_4Cl plus glutamic acid).

In the sulphate (SO_4^-) and phosphate (PO_4^-) depleted cultures, the excess was in the range of 2 - 3 fold. Since glucose and NH_4^+ were used in preference to glutamic acid (Figures 30 and 32), it is possible that the bulk of glutamic acid included in the sporulation medium remained unconsumed during sporulation (with the possible exception of C-, N- and Mg-C- cultures). This is in agreement with the finding of Buono et al. (1966). These workers found in synthetic medium, a high level of glutamic acid (70 mM) was required for the optimal growth and glucose oxidation of B. cereus followed by sporulation even though relatively little glutamic acid was actually consumed (14 mM). The reason for this excess requirement of glutamic acid remains obscure. Glutamic acid has been reported to serve as an amino donor in sporulating cells (Siegenthaler and Hermier, 1964), as a precursor to D.P.A. biosynthesis (Martin and Foster, 1958) and as a carbon and nitrogen substrate for the syntheses of other amino acids (Bernlohr, 1965). Grelet (1957) proposed that excess glutamic acid was required to counteract the injurious effects of other amino acids on sporulation. In the present study, this phenomenon cannot be confirmed in Bacillus stearothermophilus NCTC 10,003 (mutant) which sporulate in medium in the absence of other amino acids apart from glutamic acid.

On the other hand, addition of glutamic acid to the sporulation medium did not significantly increase the percentage sporulation of B. stearothermophilus NCIB 8919. The same effect was reported in B. megaterium by Hodges (1973) who also noted that spores produced in the presence of glutamic acid appeared to be brighter and larger

than those produced in its absence.

It seems therefore, that different bacteria species may respond differently to the addition of glutamic acid to sporulation medium.

D. THE MANGANESE REQUIREMENT FOR SPORULATION

The concentration of 1.0×10^{-4} M. reported here for the optimal sporulation of *B. stearothermophilus* NCTC 10,003 (mutant) is high compared with the finding of Weinberg (1964) who stated that concentrations in the range of 10^{-6} to 10^{-5} M. were required for the optimal sporulation of *Bacillus* species.

The specific manganese requirement for sporulation has been known for a very long time (Charney *et al.*, 1951), but its biochemical basis remains unknown. Weinberg (1970) has discussed possible roles of manganese in the synthesis of "secondary metabolites" during stationary phase metabolism and sporulation. It was stated that some critical biochemical processes within the sporulating cells required a high manganese level and in the absence of sufficient external manganese, the intracellular manganese drop below the critical value. Cells in the stationary phase that are not sporulating because of low manganese can be induced to sporulate by adding 10^{-5} M. manganese (Eisenstadt, 1972) and the immediate effect of adding manganese is a dramatic increase in the rate of functioning of potassium transport system which raises the internal potassium levels sufficiently to stimulate protein synthesis (Lubin, 1964; Lubin and Ennis, 1964).

In addition to the requirement for potassium maintenance,

manganese was found in complexes with small-molecular weight cofactors such as adenosine triphosphate, interactions with nucleic acids or nucleoprotein complexes such as the ribosomes. The roles of manganese in sporulation specific enzymes was studied by Eisenstadt et al., (1973). Glutamine synthetase, R.N.A. polymerase and pyruvate kinase were the three enzymes cited. When the three enzymes were changed from the magnesium to the manganese form, striking changes in the kinetic properties of the enzymes and changes in regulatory effects of substrates and feedback inhibitors were obtained. It was suggested that conversion of some key sporulation enzymes such as glutamine synthetase, pyruvate kinase and R.N.A polymerase from a predominantly magnesium form to a predominantly manganese form may be one of the roles of manganese in sporulation.

5. SPORE PROPERTIES AND COMPOSITION OF THE SPORULATION MEDIUM

A. THE EFFECT OF SPECIFIC NUTRIENT DEPLETION

Grelet (1957) indicated the possibility that the properties of the spores may be dependent upon the nature of the medium component limiting vegetative growth. Unfortunately, this finding does not seem to have had the impact it should have had, for many of the published results on bacterial sporulation rarely defined the nature of growth depletion.

Purves and Parker (1973) found that the nature of the sporulation medium affects the rate of germination of spores and the type of emergence. The nature of nutrient depletion was not determined. Spores of B. megaterium produced in a complex medium were reported to show absorption of their coats on germination whereas those produced in a chemically defined medium emerged by rupture of the coat. Also, spores produced on a complex medium were more resistant to chlorocresol than spores prepared from chemically defined medium.

Hodges and Brown (1975) produced B. megaterium spores from simple salts media in which glucose alone (G-) and glucose and magnesium were simultaneously depleted (G-Mg-). G-Mg- spores were reported to be larger than G- spores. Spores produced under the two nutrient depletion conditions also possessed different degrees of heat resistance and germination characteristics.

The results obtained with B. stearothermophilus NCTC 10,003

(mutant) are in agreement with those reported by Hodges and Brown (1975). Spores prepared from media under the conditions of nitrogen depletion (N-) and magnesium, carbon depletion simultaneously (Mg-C-) are far more heat resistant than spores produced under the condition of carbon depletion (C-) or phosphate depletion (PO_4^-). SO_4- spores possess intermediate degree of heat resistance but are extremely dormant and required prior heat activation for completed germination.

The reason for the extreme heat resistance of N- spores is not known. At a concentration of 3×10^6 spores/ml., these spores were not completely eliminated upon heating at 110°C for 2½ hr. or 121°C for 15 mn. (procedure cited by Pharmaceutical Handbook, (1970) for moist heat sterilization). Nickerson et al. (1974) prepared spores of B. thuringiensis from a glucose-glutamate-mineral salts medium in which this medium contained 0.016% glutamate (low glutamate) and 0.2% glutamate (high glutamate). Refractile spores were formed in both media, but spores prepared from low glutamate medium were extremely heat sensitive compared with spores from high glutamate medium. The heat sensitive spores were low in D.P.A. content. Further studies using radio-respirometric technique showed that cells from low glutamate medium sporulated without concurrent derepression of the T.C.A. cycle. It was concluded that a functional T.C.A. cycle may be important to D.P.A. synthesis because aspartate is a precursor of D.P.A. The N- spores used in the present study were obtained under condition where growth was terminated by the depletion of glutamic acid used as a source of nitrogen. These spores did not show any

reduction in the content of dipicolinic acid when compared with other spore types; the content of dipicolinic acid was in fact slightly elevated (Table 22). This indicates that the biosynthesis of dipicolinic acid was not impaired and a functional T.C.A. cycle was operating inspite of the depletion of glutamic acid used as a source of nitrogen.

Caslavska et al. (1972) reported cells of B. subtilis A32 grown in a chemostat under nitrogen limitation condition display morphological changes after long term cultivation. Bacillary rods were transformed into irregular spherical forms. The two morphological types differed chemically in the levels of proteins, polysaccharides and lipids. Electron-microscopic study of ultrathin sections of spherical cells showed differences in the arrangement of the superficial layers and altered cell wall was implicated. Similar detailed determination of the ratios of the individual chemical components of the bacterial wall due to the limitation of PO_4^{3-}, K^+ or Mg^{2+} in a chemostat was described by Tempest et al. (1968) and by Ellwood and Tempest (1969). It was shown that the wall was one of the phenotypically most variable structures of the cell as to chemical composition. Changes in the phospholipid composition of plasma membrane under different cultivation temperatures had also been shown in facultatively thermophilic Bacillus stearothermophilus B65 (Lee and Oo, 1973). At 37°C, phosphatidylglycerol was the major phospholipid but at 55°C, it was replaced by cardiolipin.

Since spore cortex and germ cell walls (both contain

peptidoglycan) may be formed from materials provided by the plasma membrane (as forespore membranes, Pearce and Fitz-James, 1971a), it is not surprising that the peptidoglycan of spores formed under specific nutrient depletion is characteristic of the nature of nutrient depletion (Table 22). However, the results of Table 22 shows no correlation between the chemical nature of the peptidoglycan and heat resistance of spores. This did not however, preclude the possibility that heat resistance may depend on the spatial arrangement of the cortex/germ cell wall in relation to other spore constituents. The cortex which takes up a great deal of the spore space was implicated in the maintenance of low spore protoplast pressure and hence heat resistance. Two schools of thought have been suggested. Lewis et al. (1960) suggested that a relatively dehydrated protoplast was produced and maintained by a contractile cortex (see Introduction). On the other hand, Gould and Dring (1974) argued that available evidence did not favour such a structure and proposed that the cortex was in fact expanded. Whatever the physical nature of the cortex may be, it is conceivable that specific nutrient depletion may somehow influence the nature of this organelle and the low protoplast pressure it generated.

The extreme dormant spores formed from growth depletion by sulphate is unique (Figure 39). These spores did not germinate in glucose-tryptone agar medium which readily germinated other spore types. Attempts to germinate SO_4- spores by using known physiological germinants such as L-alanine and adenosine were equally unsuccessful. Depletion of sulphate from the medium may affect

bacterial spore coats which contain proteins rich in sulphur, mostly as cysteine and cystine (Kadota et al., 1965; Kondo and Foster, 1967; Aronson and Fitz-James, 1968). These high sulphur proteins, analogous to keratins, have been implicated in the resistance of spores to heat, radiation and mechanical disruption (Vinter, 1961). It has also been suggested that the high sulphur proteins are involved in spore dormancy since reductive cleavage of disulphides in the coat proteins could initiate the germination process (Vinter, 1961). Conceivably, a distinct spore coat was formed under the condition of sulphate depletion. This coat somehow alters spore permeability to germinants. The response of SO_4- spores to reversible heat activation showed that some proteins, probably coat protein may be involved in the maintenance of the dormant state. Keynan et al. (1964) has suggested at the molecular level, heat activation is the reversible denaturation of protein. Alternatively, heating may dissociate complexes inhibitory to germination or the release of certain substances within the spore, which interact with exogenously added compounds to stimulate germination. This hypothesis is closely related to the postulated internal release of germination stimulating substances through the mediation of proteases (Levinson, 1957).

In conclusion, it seems possible to produce chemically typical spores whose major chemical components differ quantitatively, if not strictly qualitatively (Table 22) by simple manipulation of the nature of nutrient depleting the growth of a sporulation culture. These spores also differ biologically in their heat resistance and germination ability.

B. THE EFFECT OF CALCIUM, MANGANESE, IRON AND SULPHATE CONCENTRATION

The heat resistance of C- spores was increased with the increase of calcium concentration from 1.0×10^{-6} M. to 1.4×10^{-4} M. (Figure 40), thus confirming the observations of Sugiyama (1951) and Slepecky and Foster (1959). Also, at progressively lower calcium concentrations, a progressive greater proportion of the spores formed were heat sensitive. This is in agreement with the previous published results of Friesen and Anderson (1974). These workers also found that cation-exchange treatment with calcium gave rise to straight line survivor curve whereas treatments with other cations gave biphasic plots similar to that of the untreated spores. This suggests that the more heat sensitive portion of the spore population may be the result of calcium deficiency in their structure. These spores may differ intrinsically from others in their ability during sporulation to accumulate calcium. Alternatively, all the spores may be genotypically similar as discussed briefly in the experimental section, only the rates of absorption of the calcium being different. In either case, it seems probable that the earliest sporulating members of the population would preferentially consume most of the available calcium and they formed the resistant portion of the population. The residual calcium served for the spore formation in the remaining cells and they are represented by the heat-sensitive fraction of the population. Consequently, when the calcium concentration in the medium was lowered, only a small fraction of sporulating members accumulated calcium to the concentration

desirable for the attainment of maximum heat resistance. The majority of the sporulating population would give rise to heat sensitive spores. This was in fact evident from the results given in Figure 40 and Table 23.

The conferring of thermostability by calcium on bacterial spores has been attributed to its ability to bind water (Curran et al., 1943) when high heat resistance of spores was believed to be due to the high percentage of bound water (Friedman and Henry, 1938). Sugiyama (1951) suggested that calcium joined two negatively charged groups on the same folded peptide chain to confer greater resistance to the thermal unfolding of the chain, thus would constitute greater resistance to the denaturation of the protein.

The reduction in percentage germination upon the increase in the concentration of medium calcium is remarkable. Since spore dormancy is not an inherent attribute to spores, but is a function of the germination medium (Keynan et al., 1961; Levinson and Hyatt, 1964). The result of the present study is applicable only to situations in which glucose-tryptone agar is used as a germination medium. Hashimoto et al. (1969) studied the germination of individual spores of B. cereus T, induced by L-alanine and adenosine. They found two distinct phases of germination. The kinetics of the reactions involved in the first and second phase of germination are quite distinguishable from one another. It was reported that in the presence of high $CaCl_2$ (0.3 to 0.5 M.) in the medium, the induction and progress of the second phase was completely blocked whereas the first phase of germination can proceed somewhat slowly. Conceivably,

the observed drop in the percentage of germination of Bacillus stearothermophilus NCTC 10,003 (mutant) spores may be due to a similar block in the germination process since these spores tended to contain higher concentrations of calcium (Table 23).

The lowering of heat resistance of C- spores with the increase in the concentration of medium manganese (1.0×10^{-4} M. and higher) observed with B. stearothermophilus NCTC 10,003 (mutant) is in agreement with those previously reported in B. megaterium by Slepecky and Foster (1959), but not by Aoki and Slepecky (1973). It is evident from Table 25 that the accumulation of manganese in spores was correspondingly higher with spores from media richer in manganese. Since the high manganese content resulted in the lowering of calcium content, it is possible that the resistance of such spores is simply a reflection of their calcium content. Slepecky and Foster (1959) provided evidence that showed calcium content of B. megaterium spores was influenced by the levels of other ions which effectively competed with calcium for accumulation. The presence of manganese-D.P.A. chelates in spores in addition to the calcium-D.P.A. chelates has been suggested on the basis of ultra-violet spectra of dry spores (Bailey et al., 1965). It may be that for heat resistance of spores, manganese-D.P.A. chelate is not as efficient as calcium-D.P.A. chelate in conferring heat resistance to spores.

It is intersting to note that Aoki and Slepecky (1973) found that heat resistance and manganese content of B. fastidiosus spores were increased with the increase of manganese concentration in the sporulation medium. The reason for this discrepancy of results

is not known. Since different bacteria species and cultural conditions were used in each case, the interpretation of which is difficult. Nevertheless, it appears likely that part of the answer may lay in the nature of nutrient depleting the growth of sporulation culture. Hodges and Brown (1975) showed that increasing manganese concentration in the growth medium of B. megaterium, resulted in the heat resistance of glucose depleted spores (G-) being reduced, whereas those from glucose and magnesium simultaneously depleted medium (G-Mg-) was increased.

The reduced percentage germination with G- spores produced in high manganese medium (1.0×10^{-4} M. and higher) obtained with B. stearothermophilus NCTC 10,003 (mutant) had been previously reported in B. megaterium (Hodges and Brown, 1975) and in Bacillus fastidiosus (Aoki and Slepecky, 1973). In the case of Bacillus fastidiosus, this took the form of an induced heat activation requirement which increased with the increase of manganese concentration in the sporulation medium.

Manganese has previously been implicated in spore germination but from the aspect of its concentration in the germination medium. Levinson and Sevag (1953) showed that manganese in the presence of glucose initiated germination of B. megaterium spores, but not of the spores of B. subtilis or B. cereus. Glucose alone was inactive. They later (Levinson and Sevag, 1954) identified a protease enzyme in B. megaterium spores; the enzyme was activated by manganese. They proposed that the activation of this enzyme leads to the release of L-alanine formed from the autodigestion of the spore

material. This release of L-alanine in turn gave rise to germination initiation.

The results of Levinson and Sevag (1953) showed that increased germination was obtained with the inclusion of higher concentration of manganese in the medium. This contrasted with the results obtained in the present study and those previously reported by Hodges and Brown (1975) and Aoki and Slepecky (1973). It seems therefore that diffrent results may be expected from increased manganese concentration during sporulation and germination.

The effect of changes in the concentration of iron on spore properties is similar to those observed with manganese. Both iron and manganese are closely related in the periodic table of elements and this similarity seems remarkable. Very little work has been done with iron particularly on its influence on sporulation and spore properties. Iron was considered not essential for the sporulation of B. anthracis in a chemically defined medium, but its presence raised the total spore yield (Brewer et al., 1946). Using purified sucrose-mineral salt medium, a requirement for iron for sporulation was demonstrated in B. megaterium (Kolodziej and Slepecky, 1964).

The increase in the heat resistance of C- spores following lowering of sulphate concentration in the medium was unexpected (Figure 48). Bacterial spores contain more sulphur than do the corresponding vegetative forms (Vinter, 1962) and much of it exists as cystine rich protein and is part of the spore coat. The role of disulphide bonds in the resistance of B. cereus spores to heat

resistance was studied by Hitchins et al. (1966). They ruptured at least 10 to 30% of the spore disulphide bonds by reducing them to thiol groups with thioglycollic acid. They found that the high content of disulphide bonds in spore coat protein did not protect spores against heat inactivation. Aronson and Fitz-James (1968) showed that spores stripped by their coats by selective extraction were still viable and heat resistance. The effect of sulphate-depleted growth on the dormancy of B. stearothermophilus spores has been discussed in Section 5.A.

The results of the present study showed that the increase of heat resistance upon lowering of sulphate concentration was accompanied by the increase in the concentration of dipicolinic acid and calcium. This may explain the increase in heat resistance. How the level of sulphate brought about this alteration in the chemical makeup of the spore is not known. Sulphate is often included in the sporulation medium as a component of $MnSO_4$, $MgSO_4$ or Na_2SO_4. Its importance in determining spore properties has often been overlooked.

6. SPORE COMPOSITION AND HEAT RESISTANCE

With the exception of D.P.A. content, the finding of a very significant relationship between calcium, magnesium content, Mg/Ca mole ratio and heat resistance has been previously reported by Murrell and Warth (1965). These workers also found a significant relationship between diaminopimelic acid content and heat resistance and interrelationships between the content of calcium, magnesium, the mucopeptide constituents and D.P.A. They suggested that the cortex may be involved in a mechanism which controls heat resistance in spores, probably by the contractile cortex system. In this system, it was assumed that the contraction of cortical polymer is brought about by some cortically located cations. Hence, chemical variations in the cortical mucopeptide and the amount of substances causing contraction would result in differences in the degree of contraction and the final water activity of the protoplast, resulting in marked differences in heat resistance. Cations were believed to cause the reduction in the electrostatic repulsion of the carboxyl groups of the mucopeptide and spores with different cation content would possess different degree of cortical contraction and heat resistance. The nature of the cation involvement in cortical contraction remained speculative. If it is calcium, it would have been neutralized by D.P.A. as strongly suggested by the 1 to 1 mole ratio of calcium to D.P.A. It cannot be magnesium because this would require the magnesium content to increase directly with heat resistance. The results did not suggest this to be the case.

Several objections to the contractile cortex theory were raised by Gould and Dring (1974, also see Introduction), they counter-proposed that the cortex was expanded. An expanded electro-negative cortex would require the absence of neutralizing cations in order to maintain expansion and pressure. D.P.A. and cations were believed to be core located.

With the isolation of heat resistant D.P.A.- negative spore mutants and the observation that the heat resistance and germinability of these spores were lost upon storage. Gould and Dring (1974) suggested that calcium-D.P.A. in spores merely acted as a calcium buffer. In this system, calcium was believed to be lost slowly by leakage from spores. Within the spore protoplast, a constant low pool concentration of calcium which has a physiological role in dormancy and heat resistance, was maintained by dissociation of the calcium-D.P.A. complex which is believed to be core located. If this is true, then the size of this "free calcium" may be proportional to the size of calcium-D.P.A. complex which showed positive correlation with heat resistance (Figures 69 and 70). It is interesting to note that El Bisi et al. (1962) showed that there were two forms of D.P.A. in spores (loosely and firmly bound) and that only the firmly bound D.P.A. determined heat resistance.

The role of magnesium is not clear. It is unlikely to displace calcium in the calcium-D.P.A. chelate as D.P.A. chelates less strongly with magnesium than with calcium (Riemann, 1963). Moreover, magnesium content was found to varies inversely with increase in

heat resistance (Figure 71). Magnesium was shown to be required for mucopeptide synthesis in B. subtilis (Best and Durham, 1964). Magnesium depleted growth of P. aeruginosa gave rise to alterations in the ultra-structure of the cell envelope and increase of resistance to lysis by E.D.T.A. (Brown and Melling, 1968; Gilleland et al., 1974). Presumably, the role of magnesium may be to influence the structure of cortex, which may through some unknown indirect means influence the heat resistance of spores. Alternatively, magnesium may be core-located and may somehow interfere with the dissociation of calcium-D.P.A. complex (if one exists). In this case, the degree of interference may be related to the content of magnesium in spores. The amount of "free calcium" would be reduced in the presence of more intrasporal magnesium and vice versa. It seems therefore, that further study on the distribution of magnesium in spores need to be done before its physiological role can be ascertained.

7. DIPICOLINIC ACID, CATIONS RELEASE AND SPORE PROPERTIES

Energies of activation of 92.5 to 148.0 k cal./mol. (Table 30) obtained for the thermal inactivation of N- spores are higher than those previously reported by Anderson and Friesen (1974) on the same bacteria. These workers obtained an energy of activation of about 80 k cal./mol. for the inactivation reaction above the transition temperature of 100 - 105°C and about 40 k cal./mol. for the reaction occurring below 100°C. This difference may be attributed to the nature of the spore crop used. Examination of the composition of the sporulation medium used by Anderson and Friesen (1974) showed that their spores were likely to be C- spores.

Anderson and Friesen (1974) attributed the inactivation reaction proceeding above the transition temperature to be the breaking of a large number of weak, probably hydrogen bonds, since the reaction was accompanied by a large increase in the entropy of activation (140 - 150 cal./mol./deg.). The inactivation of protein molecules was implicated. If this is so, then the nature of postulated protein molecules may be determined by the nature of nutrient depletion, with the postulated proteins from N- spores being more heat resistant than proteins from C- spores. Since the energy of activation increased directly with the increase in the concentration of calcium in the medium (Figure 55), it seems probable that the ability of this protein to resist heat denaturation is to some extent determined by its association with calcium. Sadoff et al. (1965) studied the stability of glucose dehydrogenase from spores of B. cereus in an effort to understand the mechanism of its heat

resistance in spores. They found the enzymic protein underwent a reversible dimer-monomer conversion as the pH was lowered from 7.5 to 6.5, with a concomittant 260-fold increase in heat resistance. In the presence of high concentration of NaCl, further increases in heat resistance at pH 6.5 to an overall million-fold was achieved. The importance of proper ionic environment on the attainment of maximum heat resistance was implicated. Stabilization of spore protein by calcium against heat denaturation has also been suggested by Sugiyama (1951) and Keynan et al. (1965).

Walker and Matches (1965) and Grecz and Tang (1970) have reported a correlation between the rate of D.P.A. release and the heat resistance of spores. The results obtained in Figure 54 and Figure 61 did support this correlation. The apparent activation energies for the thermal inactivation of spores and that for the release of D.P.A. are however different (Table 30 and Table 32).

The apparent activation energies of D.P.A. release and dormancy breaking measured in terms of increase in colony counts of SO_4^- spores are similar (Figure 68). This is in agreement with the results previously reported by Brown and Melling (1973). They went on to propose that the nature of D.P.A. bonds, rather than the amount of D.P.A. was a determining factor for dormancy and heat resistance and that breaking of dormancy involves essentially the rupture of these D.P.A. bonds. The results of the present study showed that the nature of D.P.A. bonds is determinative in spore dormancy but not in heat resistance which probably is determined by the protein configuration mentioned above. Thus, the mechanism

whereby spores resist heat inactivation and that for the maintenance of dormancy need not be similar.

Energies of activation for the release of manganese and magnesium and that for the germination rate are all within the range expected for an enzymatic reaction (Figures 58, 65, 66 and 68). Additional informations is certainly required to pinpoint whether these events are interrelated.

8. HEAT-INDUCED DORMANCY OF SPORES OBTAINED FROM SULPHATE DEPLETED CULTURES

The finding of heat-induced dormancy when SO_4^- spores were subjected to sublethal temperatures of 95°C or less was unexpected. Similar decreases in plate counts when spores of B. stearothermophilus were subjected to sublethal temperatures has been reported by Finley and Fields (1962). These workers used spores prepared from nutrient agar incubated at 52°C for 6 days. Greater heat-induced dormancy was obtained in strain 1518 than in strain M; indicating that the degree with which heat-induced dormancy was produced was strain dependent. The present study showed that within strains, the phenomenon may also be determined by the method of cultivation, particularly the nature of nutrient depleting the growth of a sporulation culture. Brachfeld (1955) has shown heat-induced dormancy to be operative in the incubation temperature zone of B. stearothermophilus.

The mechanisms by which heat-induced dormancy operate are not known. Most of the reports cited have been shown in Bacillus stearothermophilus. An interesting finding was a report by Jaye and Ordal (1966) on B. megaterium, in which spores heated at 60°C in the presence of calcium-D.P.A. became dormant and failed to germinate in a solution of 40 mM. calcium-D.P.A., which readily germinated the unheated control. Spores which failed to germinate in calcium-D.P.A., however, responded to a germination stimulant of n-dodecylamine. The effect of heat activation on these dormant spores was not studied. It is not known whether the observed

phenomenon can be called heat-induced dormancy since spores heated at 60°C in the absence of calcium-D.P.A. did not become dormant. In the present study, heat-induced dormancy was readily demonstrated in SO_4- spores. Since sulphate depletion is likely to affect the structure of sulphur-containing protein in the spore coat, the possibility that spore dormancy is due to spore coat protein modification has been briefly discussed in Section 5. A. Other possibilities such as the change in germination requirements upon sublethal heat treatment however, cannot be ruled out. The use of other germinants apart from L-alanine and adenosine was not tried, whether or not the dormant spores respond to n-dodecylamine is not known. In any case, a satisfactory explanation of the mechanism should take into consideration the reversibility between heat-induced dormancy and heat activation (Table 38).

SUGGESTIONS FOR FURTHER WORK

1. To screen for more strains of B. stearothermophilus capable of growth in simple defined medium of glucose-mineral salts and eventually to establish the taxonomy of B. stearothermophilus capable of growing in medium containing only glucose-mineral salts.

2. Electron-microscopic examination of the structure of spores obtained from cultures variously nutrient depleted. A detailed chemical analysis of other components (such as amino acids, lipids) of spores obtained from various nutrient depleting cultures should also be made. There may be structural and chemical modifications which can be correlated with the nature of nutrient depletion.

3. To explore the possibility of producing physiologically defined spores of B. stearothermophilus by continuous cultivation using chemically defined medium. Alternatively, to cultivate physiologically defined vegetative cells by continuous cultivation and then these cells are induced to sporulate experimentally e.g. by endotrophic sporulation or microcycle sporulation.

4. To explore the use of physiologically defined spores obtained either by batch or by continuous cultivation as biological controls in sterilization processes.

5. Studies into the mechanisms governing heat-induced dormancy in B. stearothermophilus spores.

REFERENCES

Alderton, G. and Snell, N. (1963). Base exchange and heat resistance in bacterial spores. Biochem. Biophys. Res. Comm., 10, 139-143.

Alderton, G., Thompson, P.A. and Snell, N. (1964). Heat adaptation and ion exchange in B. megaterium spores. Science (New York), 143, 141-143.

Allen, M.B. (1950). The dynamic nature of thermophily. J. gen. Physiol., 33, 205-214.

Allen, M.B. (1953). The thermophilic-aerobic, spore-forming bacteria. Bact. Rev., 17, 125-173.

Amaha, M. and Sakaguchi, K. (1952). Nutritional requirements of vegetative cells and spores of aerobic spore-forming bacilli. J. Agr. Chem. Soc., Japan, 26, 353-359. Cited by O'Brien and Campbell (1957).

Amaha, M. and Sakaguchi, K. (1957). The mode and kinetics of death of the bacterial spores by moist heat. J. appl. Microbiol., 3, 163-192.

Amaha, M., Ordal, Z.J. and Touba, A. (1956). Sporulation requirements of B. coagulans var thermoacidurans in complex media. J. Bact., 72, 34-41.

Anand, J.C. (1961). J. Sci. Ind. Res. 20c, 295-298. Cited by Russell (1971).

Anderson, A.A. and Michener, H.D. (1950). Fd. Technol., 4, 188-189. Cited by Russell (1971).

Anderson, R.A. and Friesen, W.T. (1972). Growth and sporulation of B. stearothermophilus in chemically defined media. Aust. J. Pharm. Sci., 1, 1-6.

Anderson, R.A. and Friesen, W.T. (1974). The thermal resistance of B. stearothermophilus spores. The effects of temperature and pH of the heating medium. Pharm. Acta. Helv., 49, 295-298.

Anson, M.L. (1938). The estimation of pepsin, trypsin, papain and cathepsin with haemoglobin. J. gen. Physiol., 22, 79-89.

Aoki, H.A. and Slepecky, R.A. (1973). Inducement of a heat shock requirement for germination and production of increased heat resistance in B. fastidiosus spores by manganese ions. J. Bact., 114, 137-143.

Aronson, A.I. and Fitz-James, P.C. (1968). Biosynthesis of bacterial spore coats. J. Mol. Biol., 33, 199-212.

Aswad, D. and Koshland, D.E. (1974). Role of methionine in bacterial chemotaxis. J. Bact., 118, 640-645.

Atkinson, A., Evans, C.G.T. and Yeo, R.G. (1975). Behaviour of B. stearothermophilus grown in different media. J. appl. Bact., 38, 301-304.

Aubert, J.P., Millet, J. and Costoriadis-May, C. (1961). Compt. Rend., 253, 1731. Cited by Schaeffer et al. (1965).

Bailey, G.F., Karp, S. and Sacks, L.E. (1965). UV-absorption spectra of dry bacteria spores. J. Bact., 89, 984-987.

Baker, D.A. and Park, R.W.A. (1975). Changes in morphology and cell wall structure that occur during growth of Vibrio species NCTC 4716 in batch culture. J. gen. Microbiol., 86, 12-28.

Baker, H., Frank, O., Pasher, I., Black, B., Hunter, S.H. and Sobotka, H. (1960). Growth requirements of 94 strains of thermophilic bacteria. Can. J. Microbiol., 6, 557-563.

Bashnagel-De-Pamphilus, J. and Hanson, R.S. (1969). Relationship between glucose utilization and growth rate of B. subtilis. J. Bact., 98, 222-225.

Berg, P.E. and Grecz, N. (1974). Relationship of dipicolinic acid content in spores of B. cereus T to UV and γ radiation resistance. J. Bact., 103, 517-519.

Bernlohr, R.W. (1965). Role of amino acids in sporulation. In Spores III, 75-87. Editors: Campbell, L.L. and Halvorson, H.O. Michigan: American Society for Microbiology.

Bernlohr, R.W. and Leitzmann, C. (1969). Control of sporulation. In The Bacterial Spore, Chapter 6, 183-213. Editors: Gould, G.W. and Hurst, A. London and New York: Academic Press.

Best, G.K. and Durham, N.N. (1964). Effect of vancomycin on B. subtilis. Arch. Biochem. Biophys., 105, 120-125.

Bhat, J.V. and Bilimoria, M.H. (1955). Problems in thermophily. J. Ind. Inst. Sci., 37, 113-115. Cited by Baker et al. (1960).

Bigelow, A.D. and Esty, J.R. (1920). J. inf. Dis., 27, 602-617. Cited by Russell (1971).

Black, S.H. and Gerhardt, P. (1961a). Permeability of bacterial spores. I. Characterization of glucose uptake. J. Bact., 82, 743-749.

Black, S.H. and Gerhardt, P. (1961b). Permeability of bacterial spores. II. Molecular variables affecting solute permeation. J. Bact., 82, 750-760.

Black, S.H. and Gerhardt, P. (1962a). Permeability of bacterial spores. IV. Water content, uptake and distribution. J. Bact., 83, 960-967.

Black, S.H. and Gerhardt, P. (1962b). Endotrophic sporulation. Ann. New York Acad. Sci., 102, 755-762.

Black, S.H., Hashimoto, T. and Gerhardt, P. (1960). Chemical reversal of the heat susceptibility and dipicolinate deficiency of spores formed "endotrophically" in water. Can. J. Microbiol., 6, 213-224.

Bouche, J.P.C. (1973). Synthesis of unmethylated deoxyribonucleic acid in a dn aB temperature-sensitive mutant of E. coli starved for methionine. J. Bact., 115, 752-761.

Brachfeld, B.A. (1955). Studies on media and heat activation for the demonstration of viability of spores of Bacillus stearothermophilus. Ph.D. thesis. University of Illinois, Urbana III. Cited by Finley and Field (1962).

Brewer, C.R., McCullough, W.G., Mills, R.C., Roessler, W.G., Herbst, E.J. and Howe, A.F. (1946). Studies on the nutritional requirements of B. anthracis. Archiv. of Biochem., 10, 65-75.

British Standard Institution. (1963). Haemocytometer counting chambers and dilution pipettes. British Standard, 748.

Britten, R.J. and McClure, F.T. (1962). The amino acid pool in E. coli. Bact. Rev., 26, 293-335.

Brown, M.R.W. and Hodges, N.A. (1974). Growth and sporulation characteristics of B. megaterium under different conditions of nutrient limitation. J. Pharm. Pharmac., 26, 217-227.

Brown, M.R.W. and Melling, J. (1967). Dipicolinic acid release and dormancy of B. stearothermophilus spores as a function of pH and temperature. Biochem. J., 106, 44p.

Brown, M.R.W. and Melling, J. (1968). Loss of sensitivity to E.D.T.A. by P. aeruginosa grown under conditions of magnesium limitation. J. gen. Microbiol., 54, 439-444.

Brown, M.R.W. and Melling, J. (1969). Role of divalent cations in the action of polymyxin B and E.D.T.A. on P. aeruginosa. J. gen. Microbiol., 59, 263-274.

Brown, M.R.W. and Melling, J. (1973). Release of dipicolinic acid and calcium and activation of B. stearothermophilus spores as a function of time, temperature and pH. J. Pharm. Pharmac., 25, 478-483.

Brown, M.R.W., Farwell, J.A. and Rosenbluth, S.A. (1969). Use of membrane filters for measurement of 260 mμ absorbing substances from bacterial cells. Analytical Biochemistry, 27, 484-491.

Bryne, A.F., Burton, T.H. and Koch, R.B. (1960). Relation of dipicolinic acid content of anaerobic bacterial endospores to their heat resistance. J. Bact., 80, 139-140.

Buchner, H. (1890). Zeutbl. Bakt. Parasitkde 8, 1. Cited by Vinter (1969).

Bucno, F., Testa, R. and Lundgren, D.G. (1966). Physiology of growth and sporulation in B. cereus. I. Effect of glutamic acid and other amino acids. J. Bact., 91, 2291-2299.

Busta, F.F. and Adams, D.M. (1972). In Appl. Microbiol., 24, 412. Cited by Gould and Dring (1974).

Busta, F.F. and Ordal, Z.J. (1964). Heat activation kinetics of endospores of B. subtilis. J. Fd. Sci., 29, 345-353.

Cadwell, D.R. and Arcand, C. (1974). Inorganic and metal-organic growth requirements of the genus Bacteriodes. J. Bact., 120, 322-333.

Campbell, L.L. and Pace, B. (1968). Physiology of growth at high temperature. J. appl. Bact., 31, 24-35.

Campbell, L.L. and Sniff, E.E. (1959). Folic acid requirement of B. coagulans. J. Bact., 78, 267-271.

Campbell, L.L.Jr. and Williams, O.B. (1953). The effect of temperature on the nutritional requirements of facultative and obligate thermophilic bacteria. J. Bact., 65, 141-145.

Campbell, L.L., Richards, C.M. and Sniff, E.E. (1965). Isolation of strains of B. stearothermophilus with altered requirements for spore germination. In Spore III, 55-63. Editors: Campbell, L.L. and Halvorson, H.O. Ann Arbor, Michigan: American Society for Microbiology.

Caslavska, J., Stastna, J. and Vinter, V. (1970). Some artificial changes of ultrastructure of B. cereus cells in different stages of post-germinative development. Folia Microbiol. Praha 15, 197.

Caslavska, J., Kodesora, J. and Horakova, I. (1972). Effect of prolonged cultivation in a chemostat under nitrogen limitation on the morphology and ultrastructure of B. subtilis. Folia Microbiol., Praha 17, 126-131.

Chan, E.C.S., Zyk, B. and Gomersall, M. (1973). Biotin deficiency in Arthrobacter globiformis: comparative cell ultrastructure and non replacement of the vitamin by structurally unrelated compounds. J. Bact., 113, 394-402.

Chao, F.C. (1957). Dissociation of macromolecular ribonucleoprotein of yeast. Arch. Biochem. Biophys., 70, 426-431.

Charney, J., Fisher, W.P. and Hegarty, C.P. (1951). Manganese as an essential element for sporulation in the genus Bacillus. J. Bact., 62, 145-148.

Church, B.D. and Halvorson, H. (1957). Intermediate metabolism of aerobic spores. I. Activation of glucose oxidation in spores of B. cereus var terminalis. J. Bact., 73, 470-476.

Church, B.D. and Halvorson, H. (1959). Dependence of the heat resistance of bacterial endospores on their dipicolinic acid content. Nature, Lond., 188, 124-125.

Cleveland, E.F. and Gilvarg, C. (1975). Selective degradation of peptidoglycan from B. megaterium spores. In Spores VI, 458-464. Editors: Gerhardt, P., Costilow, R.N. and Sadoff, H.L. Washington, D.C: American Society for Microbiology.

Cleverdon, R.C., Pelczar, M.J. and Doetsch, R.N. (1949a). Vitamin requirements of B. coagulans. J. Bact., 58, 113-114.

Cleverdon, R.C., Pelczar, M.J. and Doetsch, R.N. (1949b). The vitamin requirements of stenothermophilic aerobic sporogenous bacilli. J. Bact., 58, 523-526.

Conway, E.J. and Downey, M. (1950). An outer metabolic region of the yeast cell. Biochem. J., 47, 347-355.

Cook, A.M. and Brown, M.R.W. (1964). The relation between heat activation and colony formation for the spores of Bacillus stearothermophilus. J. Pharm. Pharmac., 16, 725-732.

Cook, A.M. and Gilbert, R.J. (1965). The effect of yeast cells in the heating medium on the heat resistance of Bacillus stearothermophilus spores. J. Pharm. Pharmac., 17, 208-218.

Cooney, J.J. and Lundgren, D.G. (1962). Studies on asporogenic mutants of B. cereus: Preliminary investigations with calcium and zinc. Can. J. Microbiol., 8, 823-833.

Cooney, P.H., Freese, E.B. and Freese, E. (1975). Commitment of B. megaterium cells to sporulation. In Spores VI, 187-194. Editors: Gerhardt, P., Costilow, R.N. and Sadoff, H.L. Washington, D.C: American Society for Microbiology.

Costin, I.D. and Grigo, J. (1974). Zbl. Bakt. Hyg. I. Abt. Orig. A. 227, 483. Cited by Heintz et al. (1976).

Coultate, T.P. and Sundaram. T.K. (1975). Energetics of Bacillus stearothermophilus growth: molar growth yield and temperature effects on growth efficiency. J. Bact., 121, 55-64.

Cox, D.P. and Hanson, R.S. (1968). Catabolite repression of aconitate hydratase in B. subtilis. Biochem. Biophys. Acta., 158, 36-44.

Crook, P.E. (1952). The effect of heat and glucose on endogenous endospores respiration utilizing a modified scholander micro-respirometer. J. Bact., 63, 193-198.

Crosby, W.H., Greene, R.A. and Slepecky, R.A. (1971). The relationship of metal content to dormancy, germination and sporulation in B. megaterium. In Spore Research 1971, 143-160. Editors: Barker, A.N., Gould, G.W. and Wolf, J. London and New york: Academic Press.

Curran, H.R. (1957). The mineral requirements for sporulation. In Spores, 1-9. Editor: Halvorson, H.O. Washington, D.C: American Institute of Biological Sciences.

Curran, H.R. and Evans, F.R. (1937). J. Bact., 34, 179-189. Cited by Russell (1971).

Curran, H.R. and Evans, F.R. (1944). Heat activation inducing germination in the spores of thermophilic aerobic bacteria. J. Bact., 47, 437.

Curran, H.R. and Evans, F.R. (1945). Heat activation inducing germination in the spores of thermotolerant and thermophilic aerobic bacteria. J. Bact., 49, 335-340.

Curran, H.R. and Evans, F.R. (1954). The influence of iron or manganese upon the formation of mesophilic aerobes in fluid organic media. J. Bact., 67, 489-497.

Curran, H.R., Brunsletter, B.C. and Myers, A.T. (1943). Spectrochemical analyses of vegetative cells and spores of bacteria. J. Bact., 45, 485-494.

Dawes, I.W. and Mandelstam, J. (1970). Sporulation of B. subtilis in continuous culture. J. Bact., 103, 529-535.

Dawes, I.W. and Thornley, J.H.M. (1970). Theoretical and experimental studies on continuous culture systems. J. gen. Microbiol., 62, 49-66.

Dawes, I.W., Kay, D. and Mandelstam, J. (1971). Determining effect of growth medium on the shape and position of daughter chromosomes and on sporulation in B. subtilis. Nature, Lond., 230, 567-569.

De Guzman, A., Fields, M.L., Humbert, R.D. and Kazanas, N. (1972). Sporulation and heat resistance of B. stearothermophilus spores produced in chemically defined media. J. Bact., 110, 775-776.

Denny, C.B., Sharpe, L.E. and Bohrer, C.W. (1961). Appl. Microbiol., 2, 108-110. Cited by Russel (1971).

Dixon, M. and Webb, E.C. (1964). Enzymes. Second edition, London: Longmans Green.

Doering, J.L. and Bott, K.F. (1972). Differential amino acid requirements for sporulation in B. subtilis. J. Bact., 112, 345-355.

Donellan, J.E., Nag, E.H. and Levinson, H.S. (1964). Chemically defined synthetic media for sporulation and for germination and growth of B. subtilis. J. Bact., 87, 332-336.

Dring, G.J. and Gould, G.W. (1975). Re-imposition of heat resistance on germinated spores of B. cereus by osmotic manipulation. Biochem. Biophys. Res. Comm., 66, 202-208.

Duncan, C.L., Labbe, R.G. and Reich, R.R. (1972). Germination of heat- and alkali- altered spores of Cl. perfringen type A by lysozyme and an initiation protein. J. Bact., 109, 550-559.

Eisenstadt, E. (1972). Potassium content during growth and sporulation in B. subtilis W 23. J. Bact., 112, 264-267.

Eisenstadt, E. and Silver, S. (1971). Changes in calcium transport and content essential to sporulation in B. subtilis. Spore Newsletter. 4, Number 4.

Eisenstadt, E., Fisher, S., Der, C.L. and Silver, S. (1973). Manganese transport in B. subtilis W 23 during growth and sporulation. J. Bact., 113, 1363-1372.

El Bisi, H.M. and Ordal, Z.J. (1956). The effect of certain sporulation conditions on the thermal death rate of B. coagulan var thermoacidurans. J. Bact., 71, 1-9.

El Bisi, H.M., Lechowich, R.V., Amaha, M. and Ordal, Z.J. (1962). Chemical events during death of bacterial endospores by moist heat. J. Fd. Sci., 27, 219-231.

Ellwood, D.C. and Tempest, D.W. (1969). Control of teichoic acid and teichuronic acid biosynthesis in chemostat cultures of B. subtilis var niger. Biochem. J., 111, 1-5.

Ellwood, D.C. and Tempest, D.W. (1972). Effects of environment on bacterial wall content and composition. Adv. Microbiol. Physiol., 7, 83-117.

Engelbrecht, H.L. and Sadoff, H.L. (1969). Properties of purine nucleoside phosphorylase from spores and vegetative cells of B. cereus and their modification by orthophosphate. J. Biol. Chem., 244, 6228-6232.

Ennis, H.L. and Lubin, M. (1965). Pre-ribosomal particles found in K-depleted cells. Biochem. Biophys. Acta., 95, 605-623.

Epstein, I. and Grossowicz, N. (1969). Prototrophic thermophilic Bacillus: Isolation, properties and kinetics of growth. J. Bact., 99, 414-417.

Esty, J.R. and Myer, K.F. (1922). J. inf. Dis., 31, 650-663. Cited by Russell (1971).

Evans, F.R. and Curran, H.R. (1943). J. Bact., 46, 513-523. Cited by Russell (1971).

Fill, A. and Branton, D. (1969). Changes in the plasma membrane of E. coli during magnesium starvation. J. Bact., 98, 1320-1327.

Finley, N. and Fields, M.L. (1962). Heat activation and heat-induced dormancy of B. stearothermophilus spores. Appl. Microbiol., 10, 231-236.

Fitz-James, P.C. (1971). Formation of protoplasts from resting spores. J. Bact., 105, 1119-1136.

Fitz-James, P.C. and Young, E. (1969). Morphology of sporulation. In The Bacterial Spore, Chapter 2, 39-72. Editors. Gould, G.W. and Hurst, A. London: Academic Press.

Flectner, V.R. and Hanson, R.S. (1969). Coarse and fine control of citrate synthetase from B. subtilis. Biochem. Biophys. Acta., 184, 252-262.

Fleming, H.P. and Ordal, Z.J. (1964). Responses of B. subtilis spores to ionic environments during sporulation and germination. J. Bact., 88, 1529-1537.

Foerster, H.F. and Foster, J.W. (1966). Endotrophic calcium, strontium and barium spores of B. megaterium and B. cereus. J. Bact., 91, 1333-1345.

Forsberg, C.W., Wyrick, P.B., Ward, J.B. and Rogers, H.J. (1973). Effect of phosphate limitation on the morphology and wall composition of B. licheniformis and its phosphoglucomutase-deficient mutants. J. Bact., 113, 969-984.

Foster, J.W. and Heiligman, F. (1949). Mineral deficiencies in complex organic media as limiting factors in the sporulation of aerobic bacilli. J. Bact., 57, 613-646.

Frank, H.A. and Campbell, L.L.Jr. (1957). The non-logarithmic rate of thermal destruction of spores of B. coagulans. Appl. Microbiol., 5, 243-248.

Franklin, J.G. and Bradley, D.E. (1957). A further study of the spores of species of the genus Bacillus in the electron microscope using carbon replica and some preliminary observation on Cl. weichii. J. appl. Bact., 20, 467-472.

Freese, E., Kloflat, W. and Galliers, E. (1970). Commitment to sporulation and induction of glucose-phosphoenolpyruvate transferase. Biochem. Biophys. Acta. 222, 265-289.

Frehel, C. and Ryter, A. (1969). Reversibilite de la sporulation chez. B. subtilis. Ann. Inst. Pasteur, 117, 297-311. Cited by Greene and Slepecky (1972).

Friedman, C.A. and Henry, B.S. (1938). Bound water content of vegetative and spore forms of bacteria. J. Bact., 36, 99-105.

Friesen, W.T. and Anderson, R.A. (1974). Effects of sporulation conditions and cation-exchange treatment on the thermal resistance of B. stearothermophilus spores. Can. J. Pharm. Sci., 9, 50-53.

Fukuda, A and Gilvarg, C. (1968). The relationship of dipicolinate and lysine biosynthesis in B. megaterium. J. Biol. Chem., 243, 3871-3876.

Gerhardt, P. and Ribi, E. (1964). Ultrastructure of the exosporium enveloping spores of B. cereus. J. Bact., 88, 1774-1789.

Germaine, G.R. and Murrell, W.G. (1974). Use of ultraviolet radiation to locate dipicolinic acid in B. cereus spores J. Bact., 118, 202-208.

Gerrard, H.N., Parker, M.S. and Porter, G.S. (1961). The effect of pipetting on the concentration of homogeneous spore suspensions. Part II. J. Pharm., Lond., 13, 405.

Gibson, T. and Gordon, R.E. (1975). Endospores forming rods and cocci. In Bergey's Manual of Determinative Bacteriology. Eighth edition. Part 15. Baltimore: The Williams and Wilkins Company.

Gilleland, H.E., Stinnelt, J.P. and Eagon, R.G. (1974). Ultrastructural and chemical alteration of the cell envelope of P. aeruginosa associated with resistance to E.D.T.A. resulting from growth in Mg^{2+} deficient media. J. Bact., 117, 302-311.

Gilvarg, C. (1958). The enzymatic synthesis of diaminopimelic acid. J. Biol. Chem., 233, 1501-1504.

Good, T.A, and Bessman, S.P. (1964). Determination of glucosamine and galactosamine using borate buffers for modification of the Elson-Morgan and Morgan-Elson reactions. Analytical Biochemistry, 9, 253-262.

Gould, G.W. and Dring, G.J. (1974). Mechanisms of spore heat resistance. In Advances in Microbial Physiology, 11, 137-164. Editors: Rose, A.H. and Wilkinson, J.F. London and New York: Academic Press.

Grecz, N. and Olejnikow-kuritza, H. (1966). Bacteriol. Proc., page 14. Cited by Levinson and Hyatt (1969).

Grecz, N. and Tang, T. (1970). Relation of dipicolinic acid to heat resistance of bacterial spores. J. gen. Microbiol., 63, 303-310.

Grecz, N., Tang, T. and Frank, H.A. (1973). Photoprotection by dipicolinate against inactivation of bacterial spores with ultraviolet light. Spore Newsletter, 4, Number 9.

Greene, R.A. and Slepecky, R.A. (1972). Mineral requirements for commitment to sporulation in B. megaterium. J. Bact., 111, 557-565.

Grelet, N. (1946). Sporulation d'une souche de B. megaterium par e puise-ment soit du fer, soit du carbone, soit de l' azote eu milieu synthetique. C.R. Acad. Sci., Paris, 222, 418. Cited by Murrell (1961).

Grelet, N. (1951). Le determinisme de la sporation de B. megaterium I. L'effet de l'epuisement de l'aliment carbone en milieu synthetique. Ann. Inst. Pasteur, 81, 430-440. Cited by Curran, H.R. (1953). In Spore , 1-9. Editor: Halvorson, H.O. Washington,D.C: American Institute of Biological Sciences.

Grelet, N. (1952). Le determinisme de la sporulation de Bacillus megaterium. II. I' effet de la penurie de constituents mineraux du mileu synthetique. Ann. Inst. Pasteur, 82, 66-77.

Grelet, N. (1957). Growth limitation and sporulation . J. appl. Bact., 20, 315-324.

Gruft, H., Buckman, J. and Slepecky, R.A. (1965). Bact., Proc. 37. Cited by Lewis, J.C. (1969). Dormancy. In The Bacterial Spores, 301-358. Editors: Gould, G.W. and Hurst, A. London and New York: Academic Press.

Guirard, B.M. and Snell, E.E. (1962). Nutritional requirements of microorganisms. In The Bacteria IV. The Physiology of growth. Chapter 2, 33-93. Editors: Gunsalus, I.C. and Stanier, R.Y. New York and London: Academic Press.

Halvorson, H.O. (1957). Rapid and simultaneous sporulation. J. appl. Bact., 20, 305-314.

Halvorson, H.O. (1965). In Function and Structure in Microorgansim, Symp. Soc. gen. Microbiol., 15, 343-363. Editors: Pollock, M.R. and Richmond, M.H. London: Cambridge University Press.

Halvorson, H. and Howitt, C. (1961). The role of D.P.A. in bacterial spores. In Spores II, 149-164. Editor: Halvorson, H.O. Minneapolis: Burgess Publishing Co.

Halvorson, H.O. and Swanson, A. (1969). Role of dipicolinic acid in the physiology of bacterial spores. In Spores IV, 121-132. Editor: Campbell, L.L. Bethesda, Maryland: American Society for Microbiology.

Han, Y.W., Schuyten, H.A., Jr. and Callihan, C.D. (1971). The combined effect of heat and alkali in sterilizing sugarcane bagasse. J. Fd. Sci., 36, 335-338.

Han, Y.W., Zhang, H.I. and Krochta, J.M. (1976). Death rates of bacterial spores: mathematical methods. Can. J. Microbiol., 22, 295-317.

Hanson, R.S. (1966). Bact. Proc., page 96. Cited by Holmes and Levinson (1967).

Hanson, R.S. (1975). Role of small molecules in regulation of gene expression and sporogenesis in bacilli. In Spores VI, 338-326. Editors: Gerhardt, P., Costilow, R.N. and Sadoff, H.L. Washington, D.C: American Society for Microbiology.

Hanson, R.S. and Mackechnie, I. (1969). Regulation of sporulation and the entry of carbon into T.C.A. cycle. In Spores IV, 196-211. Editor: Campbell, L.L. Bethesda, Maryland: American Society for Microbiology.

Hanson, R.S., Srinivasan, V.R. and Halvorson, H.O. (1963). Biochemistry of sporulation II. Enzymatic changes during sporulation of B. cereus. J. Bact., 86, 45-50.

Hanson, R.S., Peterson, J.A. and Yousten, A.A. (1970). Unique biochemical events in bacterial sporulation. Ann. Rev. Microbiol., 24, 53-90.

Hanson, R.S., Curry, M.V., Garner, J.V. and Halvorson, H.O. (1972). Mutants of B. cereus strain T that produce thermoresistant spores lacking dipicolinate and have low levels of calcium. Can. J. Microbiol., 18, 1139-1143.

Hardwick, W.A. and Foster, J.W. (1952). On the nature of sporogenesis in some aerobic bacteria. J. gen. Physiol., 35, 907-927.

Harrell, W.K. and Mantini, E. (1957). Studies on dipicolinic acid in spores of B. cereus var terminalis. Can. J. Microbiol., 3, 735-739.

Hashimoto, T., Black, S.H. and Gerhardt, P. (1960). Development of fine structure, thermostability and dipicolinate during sporogenesis in a Bacillus. Can. J. Microbiol., 6, 203-212.

Hashimoto, T., Frieben, W.R. and Conti, S.F. (1969). Microgermination of B. cereus spores. J. Bact., 100, 1385-1392.

Hayward, A.E. (1943). Some physiological factor in spore production. J. Bact., 45, 200

Heintz, M.T., Urban, S., Schiller, I., Gay, M. and Buhlmann. (1976). The production of spores of B. stearothermophilus with constant resistance to heat and their use as biological indicators during the development of aqueous solutions for injection. Pharm. Acta. Helv., 5, 137-143.

Henning, W.A., Stern, H.S. Lanza, R.J. and Marthy, Y.S. (1973). Bull. parent. Drug Ass. 27, 30. Cited by Heintz et al. (1976).

Hills, G.M. (1949). Chemical factors in the germination of spore-bearing aerobes. Biochem. J., 45, 353-362.

Hitchins, A.D. and Gould, G.W. (1964). Release of cores from bacterial spores by mechanical breakage in acidic media. Nature, Lond., 203, 895-896.

Hitchins, A.D., King, W.L. and Gould, G.W. (1966). Role of di-sulphide bonds in the resistance of B. cereus spores to gamma irradiation and heat. J. appl. Bact., 29, 505-511.

Hitchins, A.D., Greene, R.A. and Slepecky, R.A. (1972). Effects of carbon source on size and associated properties of B. megaterium spores. J. Bact., 110, 392-401.

Hoch, J.A. and Spizizen, J. (1969). Genetic control of some early events in sporulation of B. subtilis 168. In Spores IV, 112-120. Editor: Campbell, L.L. Bethesda, Maryland: American Society for Microbiology.

Hodges, N.A. (1973). Effect of sporulation conditions on chemical composition and biological properties of B. megaterium spores. Ph. D. thesis. University of Aston in Birmingham.

Hodges, N.A. and Brown, M.R.W. (1975). Properties of B. megaterium spores formed under conditions of nutrient limitation. In Spores VI, 550-554. Editors: Gerhardt, P., Costilow, R.N. and Sadoff, H.L. Washington, D.C: American Society for Microbiology.

Holmes, P.K. and Levinson, H.S. (1967). Metabolic requirements for microcycle sporogenesis of B. megaterium. J. Bact., 94, 434-440.

Horiuchi, T. (1959). R.N.A. degradation and D.N.A. and protein synthesis of E. coli B in phosphate deficient medium. J. Biochem., (Tokyo) 46, 1467-1480. Cited by Mallette et al. (1964).

Horn, H. and Machmerth, R. (1973). Zbl. Pharm., 112, 919. Cited by Heintz et al. (1976).

Hou, C.I., Groulund, A.F. and Campbell, J.J.R. (1966). Influence of phosphate starvation on cultures of P. aeruginosa. J. Bact., 92, 851-855.

Humphrey, A.E., Kitai, A. and Cooney, C.L. (1966). Dynamics of bacterial spore cultivation in batch and continuous culture. J. Ferm. Technol., 44: 283-300.

Hutchison, K. and Hanson, R.S. (1974). Adenine nucleotide changes associated with the limitation of sporulation in B. subtilis. J. Bact., 119, 70-75.

Irr, J.D. (1972). Control of nucleotide metabolism and ribosomal ribonucleic acid synthesis during nitrogen starvation of E. coli. J. Bact., 110, 559-561.

Jacob, F. and Monod, J. (1961a). Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol., 3, 318-356.

Jacob, F. and Monod, J. (1961b). On the regulation of gene activity. Cold Spring Harb. Symp. Quant. Biol., 26, 193-211.

Jacobsen, A. and Gillespie, D. (1968). Metabolic events occurring during recovery from prolonged glucose starvation in E. coli. J. Bact., 95, 1030-1039.

Jay, M. and Ordal, Z.J. (1966). Germination response of spores of B. megaterium after exposure to calcium dipicolinate at 60°C. Can. J. Microbiol., 12, 199-201.

Kadota, H., Iijima, K. and Uchida, A. (1965). The presence of keratin like substance in spore coat of B. subtilis. Agr. Biol. Chem., 29, 870-875.

Katznelson, H. and Lochead, A.G. (1947). Nutritional requirements of B. alvei and B. paraalvei. J. Bact., 53, 83-88.

Kavanagh, F. (1960). A commentary on microbial assay. In Adv. in appl. Microbiol. 2, 65-93. Editor: Umbreit, W.W. New York and London: Academic Press.

Kay, W.W. and Groulund, A.F. (1969). Influence of carbon or nitrogen starvation on amino acid transport in P. aeruginosa. J. Bact., 100, 276-282.

Kelsey, J.C. (1958). Lancet i, 306-309. Cited by Russell (1971).

Kelsey, J.C. (1961). J. Clin. Path., 14, 313. Cited in The Catalogue of The National Collection of Type Cultures. 1972. London: Her Majesty's stationery Office.

Kennedy, R.S., Malveaux, F.J. and Cooney, J.J. (1970). Effect of glutamic acid on sporulation of B. cereus and on spore properties. Can. J. microbiol., 17, 511-519.

Kennell, D. and Kotoulas, A. (1967). Magnesium starvation of A. aerogenes. J. Bact., 93, 334-344 & 345-356.

Kennell, D. and Magasanik, B. (1962). The relation of ribosome to the rate of enzyme synthesis in A. aerogenes. Biochem. Biophys. Acta., 55, 139-151.

Kennell, D. and Magasanik, B. (1964). The control of the rate of enzyme synthesis of A. aerogenes. Biochem. Biophys. Acta., 81, 418-434.

Kerravala, D.J., Srinivasan, V.R. and Halvorson, H.O. (1964). Endogenous factor in sporogenesis in bacteria. II. Growth and sporulation in B. subtilis. J. Bact., 88, 374-380.

Keynan, A., Murrell, W.G. and Halvorson, H.O. (1961). Dipicolinic acid content, heat activation and the concept of dormancy in bacterial endospores. Nature, Lond., 192, 1211-1212.

Keynan, A., Evenchik, Z., Halvorson, H.O. and Hastings, J.W. (1964). Activation of bacterial endospores. J. Bact., 88, 313-318.

Keynan, A., Issahary-Brand, G. and Evenchik, Z. (1965). Activation of bacterial spores. In Spores III, 180-187. Editors: Campbell, L.L. and Halvorson, H.O. Ann. Arbor, Michigan: American Society for Microbiology.

Knaysi, G. (1945). A study of some environmental factors which control endospore formation in a strain of B. mycoides. J. Bact., 49, 473-493.

Knaysi, G. (1948). The endospores of bacteria. Bact. Rev., 12, 19-77.

Knaysi, G. (1965). Further observation on the spodogram of B. cereus endospores. J. Bact., 90, 453-455.

Knight, B.C.J.G. and Proom, H. (1950). A comparative survey of the nutrition and physiology of mesophilic species in the genus Bacillus. J. gen. Microbiol., 4, 508-538.

Kolodziej, B.J. and Slepecky, R.A. (1964). Trace metal requirements for sporulation of B. megaterium. J. Bact., 88, 821-830.

Komemushi, S., Takada, N. and Terni. (1966). On the change of death rate constant of bacterial spores in the course of heat sterilization. J. Ferment. Technol., 44, 543-551. Cited by Levinson and Hyatt (1971).

Kondo, M. and Foster, J.W. (1967). Chemical and electron microscopic studies on fractions prepared from coats of Bacillus spores. J. gen. Microbiol., 47, 257-271.

Kondo, M., Sano, K., Nakashio, S. and Ichikawa, T. (1975). Amino acids occurring during sporulation of B. subtilis cells. In Spores VI, 397-403. Editors: Gerhardt, P., Costilow, R.N. and Sadoff, H.L. Washington, D.C: American Society for Microbiology.

Kornberg, A., Spudich, J.A., Nelson, D.L. and Deutscher, M.P. (1968). Origin of protein in sporulation. Ann. Rev. Biochem., 37, 51-78. Editors: Boyer, P.D., Meister, A., Sinsheimer, R.L. and Snell, E.E. Palo Alto, California: Annual Reviews Inc.

Leadbetter, E.R. and Holt, S.C. (1969). Electron microscopy of frozen-etched Bacillus species. In Spore IV, 39-46. Editor: Campbell, L.L. Bethesda, Maryland: American Society for Microbiology.

Leahy, J.J. (1968). Diauxic used as a microbiological assay for L-isoleucine or L-valine in E. coli. J. Bact., 95, 1964-1965.

Leanz, G. and Gilvarg, C. (1973). Dipicolinic acid location in intact spores of B. megaterium. J. Bact., 114, 455-456.

Lechowich, R.V. (1959). Studies on thermally induced changes in the bacterial endospores and on the relationship of its chemical composition to thermal resistance. Thesis for Ph. D. in Food Tech. University of Illinois. Cited by Murrell (1961).

Lechowich, R.V. and Ordal, Z.J. (1962). The influence of the sporulation temperature on the heat resistance and chemical composition of bacterial spore. Can. J. Microbiol., 8, 287-295.

Lee, Y.H. and Oo, K.C. (1973). Metabolism of phospholipids in Bacillus stearothermophilus. J. Biochem., (Tokyo) 74, 615-621.

Leifson, E. (1931). Bacterial spores. J. Bact., 21, 311.

Levinson, H.S. (1957). Non-oxidative enzymes of spore extracts. In Spores, 120-143. Editor: Halvorson, H.O. Washington: American Institute of Biological Sciences.

Levinson, H.S. (1961). Germination factors in spore formers. In Spore II, 14. Editor: Halvorson, H.O. Minneapolis: Burgess Publishing Co.

Levinson, H.S. and Hyatt, M.T. (1960). Some effects of heat and ionizing radiation on spores of B. megaterium. J. Bact., 80, 441-451.

Levinson, H.S. and Hyatt, M.T. (1964). Effect of sporulation medium on heat resistance, chemical composition and germination of B. megaterium spores. J. Bact., 87, 876-886.

Levinson, H.S. and Hyatt, M.T. (1969). Heat activation kinetics of B. megaterium spores. Biochem. Biophys. Res. Comm., 37, 909-916.

Levinson, H.S. and Hyatt, M.T. (1970). Activation energy for glucose induced germination of B. megaterium spores. J. Bact., 103, 269-270.

Levinson, H.S. and Hyatt, M.T. (1971). Distribution and correlation of events during thermal inactivation of B. megaterium. J. Bact., 108, 111-121.

Levinson, H.S. and Sevag, M.G. (1953). Stimulation of germination and respiration of the spores of B. megaterium by manganese and monovalent anions. J. gen. Physiol., 36, 617-629.

Levinson, H.S. and Sevag, M.G. (1954). Manganese and the proteolytic activity of spore extracts of B. megaterium in relation to germination. J. Bact., 67, 615-616.

Lewis, J.C., Michener, H.D., Stumbo, C.R. and Titus, D.S. (1954). J. Agr. Fd. Chem., 2, 298-302. Cited by Russell (1971).

Lewis, J.C., Snell, N.S. and Burr, H.K. (1960). Water permeability of bacterial spores and the concept of a contractile cortex. Science, 132, 544-545.

Lewis, J.C., Snell, N.S. and Alderton, G. (1965). Dormancy and activation of bacterial spores. In Spores III, 47-54. Editors: Campbell, L.L. and Halvorson, H.O. Ann Arbor, Michigan: American Society for Microbiology.

Long, S.K. and Williams. O.B. (1958). Method for removal of vegetative cells from bacterial spore suspension. J. Bact., 76, 332

Long, S.K. and Williams, O.B. (1960). Factors affecting growth and spore formation of B. stearothermophilus. J. Bact., 79, 625-628.

Lubin, M. (1964). Intracellular potassium and control of protein synthesis. Fed. Proc., 23: 994-1001.

Lubin, M. and Ennis, H.L. (1964). On the role of intracellular potassium in protein synthesis. Biochem. Biophys. Acta., 80, 614-631.

Lund, A. (1958). Hormel Institute Ann. Rept. page 16. Cited by Murrell (1967).

Lund, A.J., Janssen, F.W. and Anderson, L.E. (1957). Effect of culture filtrates on sporogenesis in a species of Clostridium. J. Bact., 74, 577-583.

Lundgren, D.G. and Cooney, J.J. (1962). Chemical analysis of asporogenic mutants of B. cereus. J. Bact., 83, 1287-1293.

Macek, T.J. (1972). Bull. parent. Drug. Ass., 26, 18. Cited by Heintz et al., (1976).

Mackechnie, I. and Hanson, R.S. (1968). Microcycle sporogenesis of B. cereus in a chemically defined medium. J. Bact., 95, 355-359.

Macrae, R.M. and Wilkinson, J.F. (1958). Poly-B-hydroxy-butyrate metabolism in washed suspensions of B. cereus and B. megaterium. J. gen. Microbiol., 19, 210-222.

Malek, I., Chaloupka, J., Vasykova, L. and Vinter, V. (1953). cs biol 2, 323. Cited by Vinter (1969).

Mallette, M.F., Cowan, C.I. and Campbell, J.J.R. (1964). Growth and survival of E. coli in medium limited in phosphate. J. Bact., 87, 779-785.

Martin, H.H. and Foster, J.W. (1958). Biosynthesis of dipicolinic acid in B. megaterium. J. Bact., 76, 167-178.

Marquis, R.E. (1968). Salt-induced contraction of bacterial cell walls. J. Bact., 95, 775-781.

Matz, L.L., Beaman, T.C. and Gerhardt, P. (1970). Chemical composition of exosporium from spores of B. cereus. J. Bact., 101, 196-201.

Mayernik, J.J. (1972). Bull. parent. Drug. Ass., 26, 205. cited by Heintz et al. (1976).

McCarthy, B.J. (1962). The effect of magnesium starvation on the ribosome content of E. coli. Biochem. Biophys. Acta., 55, 880-888.

McGrew, S.B. and Mallette, M.F. (1962). Energy of maintenance in E. coli. J. Bact., 83, 844-850.

McIntosh, J. and Selbie, F.R. (1937). Brit. J. exp. Path., 18, 162. Cited by Powell, J.F. and Hunter, J.R. (1956). Adenosine deaminase and ribosidase. Biochem. J., 62, 381-387.

Meynell, G.G. and Meynell, E. (1970). Theory and practice in experimental bacteriology. Second edition, 1-34. London: Cambridge University Press.

Migula, W. (1904). Allegemeine Morphologie, Entwicklungsgeschi chte, Anatomie und, Systematik der Schizomyceteu. IN Lafar: Handb. techn. Mykol., 1, 29. Cited by Knaysi (1948).

Moats, W.A. (1971). Kinetics of thermal death of bacteria. J. Bact., 105, 165-171.

Moberly, B.J., Shafa, F. and Gerhardt, P. (1966). Structural details of anthrax spores during stages of transformation into vegetative cells. J. Bact., 92, 220-228.

Monod, J. (1942). Recherches sur la croissauces des cultures bacteriennes. Hermann. and cie, Paris. Cited by Paigen and Williams (1970).

Monro, R.E. (1961). Protein turnover and the formation of protein inclusions during sporulation of B. thuringiensis. Biochem. J., 81, 225-232.

Moore, W.J. (1962). Physical chemistry. Fourth edition. London: Longman Green.

Morris, D.W. and DeMoss, J.A. (1966). Polysome transitions and the regulation of ribonucleic acid synthesis in E. coli. Proc. Natl. Acad. Sci., U.S.A., 56, 262-268.

Murrell, W.G. (1961). Spore formation and germination as a microbial reaction to the environment. In Microbial Reaction to Environment, 100-150. London: Cambridge University Press.

Murrell., W.G. (1967). The biochemistry of the bacterial endospore. In Advances in Microbial Physiology, 1, 133-251. Editors: Rose, A.H. and Wilkinson, J.F. London and New York: Academic Press.

Murrell, W.G. (1969). Chemical composition of spores and spore structures. In The Bacterial Spore, 215-273. Editors: Gould, G.W. and Hurst, A. London: Academic Press.

Murrell, W.G. and Scott, W.J. (1966). Effect of moisture on heat resistance. J. gen. Microbiol., 43, 411-425.

Murrell, W.G. and Warth, A.D. (1965). Composition and heat resistance of bacterial spores. In Spores III, 1-24. Editors: Campbell, L.L. and Halvorson, H.O. Ann Arbor, Michigan: American Society for Microbiology.

Murrell, W.G., Ohye, D.F. and Gordon, R.A. (1969). Cytological and chemical structure of the spore. In Spores IV, 1-19. Editor: Campbell, L.L. Maryland: American Soc. for Microbiol.

Mychajlonka, M., Slee, A.M. and Slepecky, R.A. (1975). Requirements for microcycle sporulation in outgrowing B. megaterium cells. In Spores VI, 434-440. Editors: Gerhardt, P., Costilow, R.N. and Sadoff, H.L. Washington, D.C: American Society for Microbiol.

Nelson, F.E. (1943). Factors which influence the growth of heat-treated bacteria. I. A comparison of four agar media. J. Bact., 45, 395-403.

Nelson, D.L., Spudich, J.A., Bonsen, P.P.M., Bertsch, L.L. and Kornberg, A. (1969). Biochemical studies of bacterial sporulation and germination. In Spores IV, 72-81. Editor: Campbell, L.L. Maryland: American Society for Microbiology.

Nickerson, K.W., De Pinto, J. and Bulla, L.A.Jr. (1974). Sporulation of B. thuringiensis without concurrent derepression of the T.C.A. cycle. J. Bact., 117, 321-323.

O'Brien, R.T. and Campbell, L.L.Jr. (1957). The nutritional requirements for germination and outgrowth of spores and vegetative cell growth of some aerobic spore forming bacteria. J. Bact., 73, 522-525.

O'Conner, R.J. and Halvorson, H.O. (1961). The role of alanine dehydrogenases in the control of germination of aerobic spores initiated by L-alanine. In Spores II, 73-100. Editor: Halvorson, H.O. Minneapolis: Burgess Publishing Co.

Oka, T., Ugadawa, K. and Kinoshita, S. (1968). Unbalanced growth death due to depletion of manganese in B. ammoniagenes. J. Bact., 96, 1760-1767.

Ordal, Z.J. (1957). The effect of nutritional and environmental conditions of sporulation. In Spore, 18-32. Editor: Halvorson, H.O. Washington, D.C: American Inst. of Biological Sciences.

Ou, L.T. and Marquis, R.E. (1970). Electromechanical interactions in cell walls of Gram-positive cocci. J. Bact., 101, 92-101.

Paigen, K. and Williams, B. (1970). Catabolite repression and other control mechanisms. In Advances in Microbial Physiology, 4, 251-324. Editors: Rose, A.H. and Wilkinson, J.F. London and New York: Academic Press.

Pearce, S.M. and Fitz-James, P.C. (1971a). Sporulation of a cortex-less mutant of a variant of B. cereus. J. Bact., 105, 339-348.

Pearce, S.M. and Fitz-James, P.C. (1971b). Some refractility in variants of B. cereus treated with actinomycin D. J. Bact., 107, 337-344.

Pelcher, E.A., Fleming, H.P. and Ordal, Z.J. (1963). Some characteristics of spores of B. cereus produced by a replacement technique. Can. J. Microbiol., 9, 251-258.

Pflug, I.J. and Schmidt, C.F. (1968). Thermal destruction of micro-organisms. In Disinfection, Sterilization and Preservation, 63-105. Editors: Lawrence, C.A. and Block, S.S. Philadelphia: Lea and Febiger.

Pharmaceutical Handbook. Eighteenth edition, 1970. Editor: Todd, R.G. London: The Pharmaceutical Press.

Piperno, J.R. and Oxender, D.L. (1968). Amino acid transport systems in E. coli K12. J. Biol. Chem., 243, 5914-5920.

Porter, G.S., Brown, M.W. and Brown, M.R.W. (1967). Polarographic determination of dipicolinic acid in the presence of bacterial spores and vegetative cells. Biochem. J., 102, 19c.

Powell, E.O. (1970). Personal communication. Cited by Dawes and Thornley. (1970).

Powell, J.F. (1950). Factors affecting the germination of thick suspensions of B. subtilis spores in L-alanine solution. J. gen. Microbiol., 4, 330-339.

Powell, J.F. and Hunter, J.R. (1953). Sporulation in distilled water. J. gen. Physiol., 36, 601-606.

Powell, J.F. and Hunter, J.R. (1955). Spore germination in the genus Bacillus: the modification of germination requirements as a result of pre-heating. J. gen. Microbiol., 13, 59-67.

Powell, J.F. and Strange, R.E. (1953). Biochemical changes occurring during the germination of bacterial spores. Biochem. J., 54, 205-209.

Powell, J.F. and Strange, R.E. (1956). Biochemical changes occurring during sporulation in Bacilli species. Biochem. J., 63, 661-668.

Pulvertaft, R.J.V. and Haynes, J.A. (1951). Adenosine and spore germination: phase-contrast studies. J. gen. Microbiol., 5, 657-663.

Purves, J. and Parker, M.S. (1973). The influence of sporulation and germination media on the development of spores of Bacillus megaterium and their inhibition by chlorocresol. J. appl. Bact., 36, 39-45.

Ramaley, R.F. and Burden, L. (1970). Replacement sporulation of Bacillus subtilis 168 in a chemically defined media. J. Bact., 101, 1-8.

Reynolds, H. and Lichenstein, H. (1952). Evaluation of heat resistance data for bacterial spores. Bact. Rev., 16, 126-135.

Ridgeway, J.D. (1958). The incidence and thermal resistance of mesophilic spores found in milk and related environments. J. appl. Bact., 21, 118-127.

Riemann, H. (1963). Germination of bacterial spores with chelators. Aerhuss Stiftbogtrykkevie. Copenhagen, 91. Cited by Levinson and Hyatt (1970).

Roberts, R.B., Cowie, D.B., Abelson, P.H., Bouton, E.T. and Britten, R.J. Carnegie Inst. Wash. Publ., 607, 331. Cited by Kennell and Kotoulas (1967).

Roberts, T.A., Gilbert, R.J. and Ingram, M. (1966). The effect of sodium chloride on heat resistance and recovery of heated spores of Clostridium sporogenes. J. appl. Bact., 29, 549-555.

Rode, L.J. and Foster, J.W. (1960a). Mechanical germination of bacterial spores. Proc. Natl. Acad. Sci., U.S.A., 46, 118-128.

Rode, L.J. and Foster, J.W. (1960b). Induced release of dipicolinic acid from spores of Bacillus megaterium. J. Bact., 79, 650-656.

Rode, L.J. and Foster, J.W. (1966). Influence of exchangeable ions on germination of bacterial spores. J. Bact., 91, 1582-1592.

Rodenberg, S.D., O'Kabe, D.J., Hackel, R.A. and Cocklin, E. (1972). Factors regulating cellular development in B. cereus T spores. In Spores V, 197-202. Editors: Halvorson, H.O., Hanson, R. and Campbell, L.L. Washington, D.C: American Society for Microbioloy.

Roth, N.G., Lively, D.H. and Hodge, H.M. (1955). Influence of oxygen uptake and age of culture on sporulation of B. anthracis and B. globigii. J. Bact., 69, 445-459.

Rowe, J.J., Goldberg, I.D. and Amelunxen, R.E. (1975). Development of defined and minimal media for the growth of Bacillus stearothermophilus. J. Bact., 124, 279-284.

Rowley, D.B. and Levinson, H.S. (1967) Changes in spores of Bacillus megaterium treated with thioglycollate at low pH and restoration of germinability and heat resistance by cations. J. Bact., 93, 1017-1022.

Rowley, D.B. and Newcomb, H.R. (1964). Radiosensitivity of several dehydrogenases and transaminases during sporogenesis of Bacillus subtilis. J. Bact., 87, 701-709.

Russell, A.D. (1971). The destruction of bacterial spores. In Inhibition and Destruction of The Microbial Cell, 451-612. Editor: Hugo, W.B. London and New York: Academic Press.

Sadoff, H.L., Bach, J.A. and Kools, J.W. (1965). Significance of multiple forms of glucose dehydrogenase in relation to its heat resistance. In Spores III, 97-110. Editors: Campbell, L.L. and Halvorson, H.O. Ann Arbor, Michigan: American Society for Microbiology.

Sadoff, H.L., Hitchins, A.D. and Celikkol, E. (1969). Properties of fructose 1,6- diphosphate aldolase from spores and vegetative cells of B. cereus. J. Bact., 98, 1208-1218.

Sadoff, H.L., Celikkol, E. and Engelbrecht, H.L. (1970). Conversion of bacterial aldolase from vegetative to spore form by a sporulation-specific protease. Proc. Natl. Aca. Sci., U.S.A., 66, 844-849.

Savage, R.H.M. (1959). In The Operation of Sterilizing Autoclaves, 1-11. London: Pharmaceutical Press.

Schaeffer, P., Millet, J, and Aubert, J.P. (1965). Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci., 54, 704-711.

Scherrer, R. and Gerhardt, P. (1972). Location of calcium within Bacillus spores by electron probe X-ray microanalysis. J. Bact., 112, 559-568.

Schreiber, O. (1896). Zentbl. Bakt. Parasitkde. 20, 353-429. Cited by Vinter (1969).

Setlow, P. (1974). Identification of several unique low molecular weight basic protein in dormant spores of B. megaterium and their degradation during spore germination. Biochem. Biophys. Res. Comm., 61, 1110-1117.

Schull, J.J. and Ernst, R.R. (1962). Appl. Microbiol., 10, 452-457. Cited by Russell (1971).

Siegenthaler, P.A. and Hermier, S. (1964). Nature des systemes enzymatiques responsables de l'utilization des acides amines sources d'azote chez B. subtilis. Ann. Inst. Pasteur, 106, 194-213.

Silverster, C.J. (1964). Nutritional requirements of Bacillus popilliae. J. Bact., 87, 114-119.

Slepecky, R.A. and Foster, J.W. (1959). Alterations in metal content of spores of B. megaterium and the effect on some spores properties. J. Bact., 78, 117-123.

Slepecky, R.A. and Law, J.H. (1960). Synthesis and degradation of poly-β-hydroxybutyric acid in connection with sporulation of B. megaterium. J. Bact., 82, 37-42.

Slepecky, R.A. and Law, J.H. (1962). Unpublished results. Cited by Halvorson, H.O. (1962). In The Bacteria IV, 223-264. Editors: Gunsalus, I.C. and Stanier, R.Y. New York and London: Academic Press.

Slock, J.A. and Stahly, D.P. (1974). Polysaccharide that may serve as a carbon and energy storage compound for sporulation in Bacillus cereus. J. Bact., 120, 399-406.

Splittstoesser, D.F. and Farkas, D.F. (1966). Effect of cations on activation of B. popilliae spores. J. Bact., 92, 995-1001.

Spudich, J.A. and Kornberg, A. (1968). Biochemical studies of bacterial sporulation and germination. J. Biol. Chem., 243, 4588-4599 & 4600-4605.

Srinivasan, V.R. (1965). Intracellular regulation of sporulation of bacteria. In Spores III, 64-74. Editors: Campbell, L.L. and Halvorson, H.O. Michigan: American Soc. for Microbiology.

Srinivasan, V.R. and Halvorson, H.O. (1963). Endogenous factor in sporogenesis in bacteria. Nature, Lond., 197, 100-101.

Stanier, R.Y., Doudoroff, M. and Adelberg, E.A. (1971). In General Microbiology, third edition, 314. London: Macmillan.

Stastna, J. and Vinter, V. (1970). Spores of microorganisms. XXIII. Interdependence of intra and extra- cellular levels of calcium: Its effects on the germination of bacterial spores in different media. Folia Microbiol., Praha, 15, 103-110.

Stearn, A.E. (1949). Kinetics of biological reactions with special reference to enzymic processes. In Adv. Enzymol., 9, 25-74.

Sugiyama., H. (1951). Studies on factors affecting the heat resistance of spores of Cl. botulinum. J. Bact., 62, 81-96.

Sundaram, T.K. (1973). Physiological role of pyruvate carboxylase in a thermophilic Bacillus. J. Bact., 113, 549-557.

Szulmajster, J. (1973). Initiation of bacterial sporogenesis. In Microbial Differentiation, 45-83. Editors: Ashworth, J.M. and Smith, J.E. London: Cambridge University Press.

Szulmajster, J. and Hanson, R.S. (1965). Physiological control of sporulation in B. subtilis. In Spores III, 162-173. Editor: Campbell, L.L. and Halvorson, H.O. Ann Arbor, Michigan: American Society for Microbiology.

Tallentire, A. and Chiori, C.O. (1963). Heat and gamma radiation resistance of B. megaterium spores. J. Pharm. Pharmac., 15, 148T-149T.

Tatum, E.L. (1946). Induced biochemical mutations in bacteria. Cold Spring Harb. Symp. Quant. Biol., 11, 278-284.

Tempest, D.W. (1970). The place of continuous culture in microbiological research. Advances in Microbial Physiology, 4, 223-250. Editors: Rose, A.H. and Wilkinson, J.F. London and New York: Academic Press.

Tempest, D.W., Dicks, J.W. and Ellwood, D.C. (1968). Influence of growth condition on the concentration of potassium in B. subtilis var niger and its possible relationship to cellular ribonucleic acid, teichoic acid and teichuronic acid. Biochem. J., 106, 237-243.

Thomas, R.S. (1964). Ultrastructural localization of mineral matter in bacterial spores by microincineration. J. Cell. Biol., 23, 113-133.

Thorpe, R.H. (1960). The action of nisin on spoilage bacteria. I. The effect of nisin on the heat resistance of Bacillus stearothermophilus spores. J. appl. Bact., 23, 136-143.

Tinelli, R. (1955). Ann. Inst. Pasteur, 88, 212. Cited by Halvorson, H. (1962). In The Bacteria IV, 223-264. Editors: Gunsalus, I.C. and Stanier, R.Y. New York and London: Academic Press.

Tipper, D.J. and Gauthier, J.J. (1972). Structure of the bacterial endospore. In Spores V, 3-12. Editors: Halvorson, H.O., Hanson, R.S. and Campbell, L.L. Washington, D.C: American Society for Microbiology.

Tono, H. and Kornberg, A. (1967). Biochemical studies of bacterial sporulation. J. Bact., 93, 1819-1824.

Umbarger, H.E. and Brown, B. (1955). Isoleucine and valine metabolism in Escherichia coli. V. Antagonism between isoleucine and valine. J. Bact., 70, 241-248.

United States Pharmacopeia XIX. Cited by Heintz et al. (1976).

Vallee, B.C. (1960). Metal and enzyme interactions: correlation of composition, function and structure. In Enzymes. Editors: Boyer, P.O., Lardy, H. and Myrback, K. New York: Academic Press.

Vas, K. (1970). Problems in thermal processing. J. appl. Bact., 33, 157-166.

Vinter, V. (1959). Differences in cysteine content between vegetative cells and spores of Bacillus cereus and Bacillus megaterium. Nature, Lond., 183, 998-999.

Vinter, V. (1961). The formation of cystine-rich structure in sporulating cells and its possible role in the resistance of spores. In Spores II, 127-141. Editor: Halvorson, H.O. Minneapolis: Burgess Publishing Co.

Vinter, V. (1962). Spores of microorganisms. IX. Gradual development of the resistant structure of bacterial endospores. Folia. Microbiol., 7, 115.

Vinter, V. (1969). Physiology and biochemistry of sporulation. In The Bacterial Spore, Chapter 3, 73-123. Editors: Gould, G.W. and Hurst, A. London and New York: Academic Press.

Vinter, V. and Slepecky, R.A. (1965). Direct transition of outgrowing bacterial spores to new sporangia without intermediate cell division. J. Bact., 90, 803-807.

Vinter, V. and Vechet, B. (1964). Spores of microorganisms. XV. The alteration of heat sensitivity and its relation to radiation resistance of bacterial spores. Folia. Microbiol., Praha., 9, 238-248.

Waites, W.M. (1968). Sporulation in Bacillus subtilis: Antigenic changes during spore formation. Biochem. J., 109, 803-810.

Walker, P.D. (1970). Cytology of spore formation and germination. J. appl. Bact., 33, 1-12.

Walker, H.W. and Matches, J.R. (1965). Release of cellular constituents during heat inactivation of endospores of aerobic bacilli. J. Fd. Sci., 30, 1029-1036.

Walker, H.W., Matches, J.R. and Ayres, J.C. (1961). Chemical composition and heat resistance of some aerobic bacterial spores. J. Bact., 82, 960-966.

Ward, B.Q. (1947). Studies on the sporulation of Bacillus thermoacidurans. Ph.D. thesis, University of Texas, 1-90. Cited by Curran (1957).

Warth, A.D. and Strominger, J.L. (1969). Structure of the peptidoglycan of bacterial spores: Occurrence of the lactam of muramic acid. Proc. Natl. Aca. Sci., U.S.A., 64, 528-535.

Warth, A.D. and Strominger, J.L. (1971). Structure of the peptidoglycan from vegetative cell walls of Bacillus subtilis. Biochemistry, 10, 4349-4358.

Warth, A.D., Ohye, D.F. and Murrell, W.G. (1963). The composition and structure of bacterial spores. J. Cell. Biol., 16, 579-592.

Weibull, C. (1956). Bacterial protoplasts; their formation and characteristics. Symp. Soc. Gen. Microbiol., 6,111-126.

Weil, R. (1899). Arch. Hyg., 35, 355. Cited by Halvorson, H. (1962). In The Bacteria IV, 223-264. Editors: Gunsalus, I.C. and Stanier, R.Y. New York and London: Academic Press.

Weinberg, E.D. (1964). Manganese requirements for sporulation and other secondary biosynthetic processes of Bacillus. Appl. Microbiol., 12, 436-441.

Weiberg, E.D. (1970). Biosynthesis of secondary metabolites: roles of trace metals. In Adv. Microbiol. Physiol., 4, 1-44. Editors: Rose, A.H. and Wilkinson, J.F. London and New York: Academic Press.

Welker, N. and Campbell, L. (1963). Effect of carbon sources on the formation of alpha-amylase by B. stearothermophilus. J. Bact., 86, 681-688.

White, P.J. (1972). The nutrition of Bacillus megaterium and Bacillus cereus. J. gen. Microbiol., 71, 505-514.

Williams, O.B. and Harper, O.F. Jr. (1951). Studies on heat resistance. IV. Sporulation of Bacillus cereus in some synthetic media and the heat resistance of the spores produced. J. Bact., 61, 551-556.

Williams, O.B. and Robertson, W.J. (1954). Studies on heat resistance. VI. Effect of temperature of incubation on heat resistance of aerobic thermophilic bacteria. J. Bact., 67, 377-378.

Willis, J.B. (1960). The determination of metals in blood serum by atomic absorption spectroscopy. Spectrochimica Acta., 16, 259-272.

Wolf, J. and Barker, A.N. (1968). The genus Bacillus: aids to the identification of its species. In Identification Methods For Microbiologists. Part B, 93-109. Editors: Gibbs, G.M. and Shapton, D.A. London and New York: Academic Press.

Wyatt, P.J. (1975). Observations on the structure of spores. J. appl. Bact., 38, 47-51.

Young, I.E. (1959). A relationship between the free amino acid pool, dipicolinic acid and calcium from resting spores of Bacillus megaterium. Can. J. Microbiol., 5, 197-202.

Young , F.E. and Wilson, G.A. (1972). Genetics of Bacillus subtilis and other Gram-positive sporulating bacilli. In Spore V, 77-106. Editors: Halvorson, H.O., Hanson, R. and Campbell, L.L. Washington, D.C: American Society for Microbiology.

Zytkovicz, T.H. and Halvorson, H.O. (1972). Some characteristics of dipicolinic acid-less mutant spores of Bacillus cereus, Bacillus megaterium and Bacillus subtilis. In Spores V, 49-52. Editors: Halvorson, H.O., Hanson, R. and Campbell, L.L. Washington, D.C: American Society for Microbiology.